Effects of Excess Selenium on the Health and Reproduction of White Sturgeon (*Acipenser transmontanus*): Implications for San Francisco Bay-Delta

by

REGINA GAIL LINVILLE
B.A. (University of California, Berkeley) 1994

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Ecology

in the

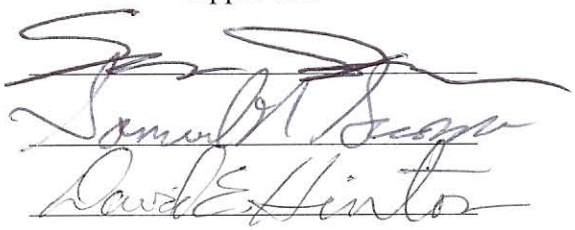
OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

Davis

Approved:



Committee in Charge

Serge I. Doroshov
Samuel N. Luoma
David E. Hinton

2006

Acknowledgements

This work has been far from a solitary effort. Contributions from mentors, colleagues, friends, and funding organizations have led to the successful completion of this dissertation. Serge Doroshov, my major advisor, has offered his constant support and direction throughout this work. I was fortunate to work with someone with such extensive knowledge and experience in fish physiology and reproduction. I have learned so much from him and will always be grateful. Dave Hinton has made invaluable contributions to this endeavor by serving on my dissertation committee and by offering his expertise in pathology and ecotoxicology. Sam Luoma has been my long-term mentor and I continually attempt to emulate him as a scientist. Sam helped me to form the thesis of this work while I was a member of his research team at the US Geological Survey. All that I learned from him there, along with his continued support and advice, has shaped every aspect of this research.

The work presented here would not have been possible without the help of Joel Van Eenennaam and Javier Linares. Their expertise in sturgeon culture, maintenance, sampling and research was critical to this project. I am extremely grateful to them for their tireless help and advice throughout this research. I am also grateful to Paul Lutes and Erik Hallen for their support and advice regarding sturgeon maintenance.

Ed DePeters has my sincere gratitude for allowing me extensive use of his lab. My work there was successful due to Scott Taylor's constant support and advice. Silas Hung also opened his lab to me, and provided critical support with the experimental diets. Silas formulated the juvenile experimental diet and directed its production. He also provided the use of his pelleting equipment for production of the adult experimental diet.

I am very grateful to Xin Deng for his collaboration in the fluorometric analyses, his help with sampling, and for our fruitful discussions on selenium research. The first chapter of this dissertation would not have been possible without David Hinton. His work on the histopathology, as well as his direction and training, was critical to that research. Similarly, Kevin Kroll's collaboration in the isolation and purification of vitellogenin and egg yolk proteins was essential to the research presented here in chapter two. A special thanks to Don Tillitt and Diana Papoulias of the US Geological Survey for providing training on the microinjection of sturgeon larvae. Javier Linares received that training and then returned to share it with the Doroshov Lab. The microinjection experiment would not have been possible without Javier's extensive collaboration.

I had the fortunate opportunity to work with several undergraduate interns including Fiona Sands, Summer Evert, Matthew Morales, Karen Kaplan, Rachel Hutchins, Blair Kendal, Carla Rodriguez, Melissa Dyer, Anita Setty, Shinichiro Oku, Michelle Jones, Todd Tolentino, Amanda Cassidy, Dominique Weddle, Angela Sartor, and Melissa Arevalo. These individuals assisted in sampling, chemical analyses, and much more. They worked hard, offered insight, and contributed a great deal to this research. I am very grateful to each of them.

Obtaining funding for graduate research is becoming increasingly difficult. I am extremely grateful to the following organizations for their financial support through fellowships, grants and contracts: the UC Toxic Substances Research and Teaching Program, the Ecotoxicology Lead Campus Program at UC Davis, the Graduate Group in Ecology, the US Geological Survey and CALFED.

During the earlier years of this endeavor, I received valuable direction and support from several faculty members of the Graduate Group in Ecology. Mike Johnson was especially helpful by continuously providing insight and serving as the chair of my qualifying examination committee. Keith Miles, Barry Wilson, and Joe Cech also contributed to my early studies and planning. I also learned much through extensive discussions during seminars and conferences hosted by the Graduate Group in Ecology, its Ecotoxicology AOE, and the Lead Campus Program in Ecotoxicology.

Most of all, I am immensely grateful for the support of family and friends throughout this arduous process. My parents, Jess and Edna Linville, have provided unwavering love and support. My sister and friend, Cynthia Linville, has offered encouragement, advice, and her expertise in the use of the English language. I am grateful to the many people that have brightened my life so much throughout these past years including Mikaela Huntzinger, Jennifer Von Aesch, Diana Rowan, Anne Salo, Scott Nagy, Marta Sykes, Dena McCann, Darcy Jones, and Hali Rowen. I give my fondest thanks and appreciation to my friend and husband, Jed Alexander, who has given his continuous love, support and encouragement.

Abstract

Selenium (Se) is a potent toxicant in aquatic ecosystems, threatening higher trophic level species because of its efficient food web transfer. Excess Se consumption in fish has led to reduced growth, significant histopathology in major organs, reproductive failure, developmental defects and mortality. Se is also an essential nutrient; however, there is a very narrow margin between nutritional and toxic concentrations of this element. White sturgeon (*Acipenser transmontanus*) in San Francisco Bay-Delta consume bivalves containing high levels of Se and exhibit elevated Se concentrations in their muscle, liver and eggs, which may threaten their health and reproduction.

Juvenile white sturgeon (47 weeks post-hatch) were exposed to dietary organic Se (as selenized yeast; *ca.* 1, 20, 36, and 53 μg/g Se) for six months. The effects of excess dietary Se included the dose-response increase of melanomacrophage aggregates in liver, the occurrence of cholestasis, a potentially fatal condition, in all treatment groups and inflammation and fused lamellae in gills. No effects were observed on the size or growth of these individuals.

Female white sturgeon, in late stage vitellogenesis, were given dietary Se (as selenized yeast; *ca.* 1 and 34 μg/g Se) for up to six months. Treatment females accumulated *ca.* 11, 10 and 12 Se μg/g (dw) in muscle, liver and eggs. Transfer of Se to the yolk in developing eggs took place through the yolk protein precursor, vitellogenin (VTG). Se uptake by VTG in the liver took place in a dose-dependent manner. Similarly, Se concentrations in eggs showed a dose-dependent relationship with Se in plasma VTG. Se in eggs was associated with the yolk proteins, lipovitellin, phosvitin and immunoglobulin.

Hormonal treatment was used to induce spawning in females. We did not observe an impact on reproduction, as shown by fecundity, fertilization success and survival to neurulation. Progeny of treatment females (embryos and yolk sac larvae) exhibited high Se concentrations (similar to egg concentrations).

Yolk utilization in larvae from treatment females led to significant skeletal deformities (lordosis, kyphosis, and scoliosis; ED_{25} = 24 μg/g Se in larvae, dw), edema (ED_{25} = 25 μg/g Se; Logit, $p < 0.0001$), and mortality (8% in larvae with *ca.* 20 μg/g Se; Kaplan-Meier, $p < 0.0001$). In addition, yolk sac larvae (from unexposed females) that were microinjected with seleno-L-methionine exhibited developmental defects including skeletal deformities (lordosis, kyphosis, and scoliosis; ED_{25} = *ca.* 17.5 μg/g Se in larvae, dw) and edema (ED_{25} = *ca.* 13 μg/g Se; Logit, $p < 0.0001$). Microinjected yolk sac larvae experienced a greater incidence of mortality (45 - 70% in larvae with *ca.* 20 μg/g Se Kaplan-Meier, $p < 0.0001$) as compared to maternally exposed yolk sac larvae. For both experimental approaches (maternal transfer and microinjection), white sturgeon yolk sac larvae containing Se concentrations above *ca.* 11 to 15 μg/g demonstrated significant increases of mortality and abnormality rates. A hazard threshold range of *ca.* 3 to 8 μg/g Se in developing white sturgeon is suggested for this species.

In San Francisco Bay-Delta, juvenile white sturgeon are susceptible to toxicity at Se concentrations currently observed in some prey items (i.e., bivalves), and accumulate Se in their tissues at concentrations similar to those observed in our study. This leads us to suspect that juvenile white sturgeon in this area may be experiencing significant liver damage, depending on individual dietary Se exposure. Adult white sturgeon in San Francisco Bay-Delta exhibit Se body burdens similar to those accumulated by treatment

fish in our adult experiment. This suggests that female white sturgeon in San Francisco Bay-Delta are likely transferring Se to their offspring. Limited data on Se concentrations in white sturgeon eggs and ovaries from this region supports this conclusion. Our research shows that elevated Se (*ca.* 11 – 15 μg/g Se) in white sturgeon eggs leads to significant developmental defects and mortality in developing larvae. Based on the work presented here, we suspect that white sturgeon recruitment in San Francisco Bay-Delta is impacted by the accumulation of excess Se in this species. The abundance of this population was recently recorded at a 50-year low. Careful management of all processes with potential to increase Se concentrations in the benthic food web is essential to protect sturgeon in San Francisco Bay-Delta and other high-Se systems.

Table of Contents

Title page i

Acknowledgements ii

Abstract v

Table of contents viii

Table of tables x

Table of figures xiii

Introduction

Selenium in San Francisco Bay-Delta 1

White sturgeon in San Francisco Bay-Delta 4

Toxicity of selenium 7

Research aims 12

Chapter 1. Toxicity of foodborne selenium to juvenile white sturgeon, with an emphasis on histopathology of the liver

Introduction 13

Materials and Methods 17

Results 22

Discussion 30

Tables and Figures 41

Chapter 2. Dose-dependent maternal transport of selenium via vitellogenin in white sturgeon

Introduction 62

Materials and Methods 65

Results 81

Discussion 86

Tables and Figures 94

Chapter 3. Developmental toxicity in white sturgeon larvae exposed to selenium through maternal transport or microinjection

Introduction 126

Materials and Methods 129

Results 141

Discussion 150

Tables and Figures 158

Conclusions 196

References 200

Appendix A. Biliary transport and cholestasis in fish 218

Appendix B. Summary data on spawning induction and success in white sturgeon fed either control (*ca.* 1.42 µg/g Se) or treatment (*ca.* 34 µg/g Se) diets for approximately six months 227

Tables 228

Table of Tables

Chapter 1. Toxicity of foodborne selenium to juvenile white sturgeon, with an emphasis on histopathology of the liver

Table 1-1. Selenium content of juvenile experimental diets 41

Table 1-2. Composition of juvenile experimental diet 42

Table 1-3. Selenium in juvenile white sturgeon after six months of dietary selenium exposures 43

Table 1-4. Comparisons of gross morphological characteristics of juvenile white sturgeon following six months of dietary selenium exposures 44

Table 1-5. Gross morphological measurements of juvenile white sturgeon following six months of dietary selenium exposure 45

Chapter 2. Dose-dependent maternal transport of selenium via vitellogenin in white sturgeon

Table 2-1. Selenium content of adult experimental diets 94

Table 2-2. Combined vitellogenin protein samples 95

Table 2-3. Morphological comparisons between exposure groups 96

Table 2-4. Selenium in adult white sturgeon after six months of dietary selenium exposures 97

Table 2-5. Percent moisture of adult white sturgeon tissues 98

Table 2-6. Selenium levels measured in plasma and isolated plasma proteins of white sturgeon after six months of dietary selenium exposures 99

Table 2-7. Selenium concentrations in yolk proteins isolated from eggs of adult white sturgeon following six months of dietary exposures 100

Table 2-8. Selenium in combined vitellogenin samples compared to combined tissue and yolk protein samples 101

Table 2-9. Statistical parameters for the regressions of selenium concentrations in ovarian tissue, eggs, plasma and non-vitellogenin isolated plasma proteins as a function of selenium concentrations in liver 102

Table 2-10. Pearson correlation coefficients for selenium in plasma non-vitellogenin proteins correlated to selenium in liver, yolk proteins, and eggs 103

Table 2-11. White sturgeon spawning data 104

Table 2-12. Reproductive performance of sturgeon females in control and treatment groups 105

Chapter 3. Developmental toxicity in white sturgeon larvae exposed to selenium through maternal transport or microinjection

Table 3-1. Selenium content of adult experimental diets 158

Table 3-2. Key developmental stages in sturgeon 159

Table 3-3. Size of larvae from control and treatment female white sturgeon 160

Table 3-4. Size of larvae in microinjection study two 161

Table 3-5. Selenium in developing white sturgeon progeny of females following dietary selenium exposure during late vitellogenesis 162

Table 3-6. Selenium in developing white sturgeon exposed to seleno-L-methionine by microinjection 163

Table 3-7. Occurrence of edema in larvae for each progeny cohort of female white sturgeon exposed to dietary selenium 164

Table 3-8. Occurrence of skeletal deformities in larvae for each progeny cohort of female white sturgeon exposed to dietary selenium 165

Table 3-9. Affected larvae (edema and/or skeletal deformities) in each progeny cohort of female white sturgeon exposed to dietary selenium 166

Table 3-10. Occurrence of developmental defects in larvae from female white sturgeon exposed to dietary selenium 167

Table 3-11. Percentage of larvae affected by edema, lordosis, kyphosis, and scoliosis from microinjection study one 168

Table 3-12. Percentage of larvae with developmental defects for each progeny cohort of microinjection study two 169

Table 3-13. Percentage of larvae affected by edema, lordosis, kyphosis, and scoliosis from microinjection study two 170

Table 3-14. Percent mortality in larvae from female white sturgeon exposed to dietary selenium 171

Table 3-15. Percent mortality in larvae exposed to selenium via microinjection 172

Table 3-16. Combined mortality in larvae from maternal exposure and microinjection studies 173

Table 3-17. Probabilities of developmental defects in larvae containing elevated selenium levels at the beginning of larval development 174

Table 3-18. Selenium in adult tissues and their progeny related to incidence of developmental defects 175

Table 3-19. Potential hazard threshold levels for selenium in developing white sturgeon larvae 176

Appendix B. Summary data on spawning induction and success in white sturgeon fed either control (*ca.* 1.42 µg/g Se) or treatment (*ca.* 34 µg/g Se) diets for approximately six months.

Table B-1. Detailed spawning data for white sturgeon fed either control (*ca.* 1.42 µg/g Se) or treatment (*ca.* 34 µg/g Se) diets for approximately six months 228

Table B-2. Summary of spawning induction and success in white sturgeon fed either control (*ca.* 1.42 µg/g Se) or treatment (*ca.* 34 µg/g Se) diets for approximately six months 232

Table of Figures

Chapter 1. Toxicity of foodborne selenium to juvenile white sturgeon, with an emphasis on histopathology of the liver

Figure 1-1. Selenium levels in liver, blood and muscle after six months of dietary selenium exposures 46

Figure 1-2. Micrograph showing overview of control liver architecture 47

Figure 1-3. Micrograph of control liver showing bile duct 48

Figure 1-4. Micrograph of control liver showing bile retention in a single control liver, with no evidence of biliary stasis 49

Figure 1-5. Micrograph of control liver showing eosinophilic granular leukocytes 50

Figure 1-6. Micrograph of control liver showing moderate inflammation 51

Figure 1-7. Micrograph of control liver showing peribiliary inflammation 52

Figure 1-8. Micrograph showing distended canalicular network in liver of low treatment fish 53

Figure 1-9. Micrograph showing tubuloform swelling in liver of low treatment fish 54

Figure 1-10. Micrograph showing bile duct proliferation in liver of low treatment fish 55

Figure 1-11. Micrograph showing bile duct proliferation with inflammation in liver of low treatment fish 56

Figure 1-12. Micrograph showing necrosis and distended canalicular network in liver of medium treatment fish 57

Figure 1-13. Micrograph showing bile duct proliferation and peribiliary inflammation in liver of medium treatment fish 58

Figure 1-14. Micrograph showing extensive melanomacrophage aggregates in liver of high treatment fish 59

Figure 1-15. Micrograph showing distended canalicular network and hepatocyte vacuolation in liver of high treatment fish 60

Figure 1-16. Contingency table showing the occurrences of inflammation and fused lamellae in gills of juvenile white sturgeon exposed to dietary selenium for six months 61

Chapter 2. Dose-dependent maternal transport of selenium via vitellogenin in white sturgeon

Figure 2-1. Hypothesized mechanism of the maternal transport of selenium to offspring 106

Figure 2-2. Anion exchange chromatograph of white sturgeon male before and after estrogen treatment 107

Figure 2-3. Anion exchange chromatograph of plasma proteins from vitellogenic white sturgeon female used for this study 108

Figure 2-4. PAGE & Western gels of white sturgeon plasma proteins 109

Figure 2-5. Vitellogenin ELISA standard curve 110

Figure 2-6. Gel filtration chromatograph of sturgeon egg yolk 111

Figure 2-7. PAGE and Western gels of crude and purified egg yolk proteins of white sturgeon 112

Figure 2-8. Tissue selenium concentrations in adult white sturgeon after *ca.* six months of dietary selenium exposures 113

Figure 2-9. Selenium in plasma collected at mid- and end-experiment 114

Figure 2-10. Selenium in vitellogenin isolated from plasma collected at mid- and end-experiment 115

Figure 2-11. Selenium in non-vitellogenin proteins isolated from plasma collected at mid- and end-experiment 116

Figure 2-12. Selenium concentrations in vitellogenin and non-vitellogenin proteins 117

Figure 2-13. Vitellogenin isolated from plasma collected at mid- and end-experiment 118

Figure 2-14. Selenium in vitellogenin related to selenium in liver 119

Figure 2-15. Selenium in plasma, ovarian tissue and eggs related to selenium in vitellogenin 120

Figure 2-16. Selenium in yolk proteins related to selenium in vitellogenin 121

Figure 2-17. Selenium in isolated yolk proteins as a function of selenium in eggs 122

Figure 2-18. Selenium in ovary, eggs, plasma and non-vitellogenin plasma proteins as a function of selenium in liver 123

Figure 2-19. Selenium in non-vitellogenin plasma proteins related to total selenium in plasma 124

Figure 2-20. Selenium in eggs related to selenium in non-vitellogenin plasma proteins 125

Chapter 3. Developmental toxicity in white sturgeon larvae exposed to selenium through maternal transport or microinjection

Figure 3-1. Selenium concentrations in eggs and developing embryos and larvae from white sturgeon females exposed to dietary selenium 177

Figure 3-2. Selenium concentrations in developing white sturgeon larvae following microinjection of seleno-L-methionine 178

Figure 3-3. Developmental defects observed in white sturgeon during larval development 179

Figure 3-4. Percentage of maternally exposed larvae with edema related to selenium concentrations in larvae 180

Figure 3-5. Percentage of maternally exposed larvae with skeletal deformities related to selenium concentrations in larvae 181

Figure 3-6. Percentage of maternally exposed larvae with edema and/or skeletal deformities related to selenium concentrations in larvae 182

Figure 3-7. Percentage of maternally exposed larvae with edema and/or skeletal deformities related to selenium concentrations in eggs 183

Figure 3-8. Percentage of larvae from microinjection study one with edema related to selenium concentrations in larvae at the onset of larval development 184

Figure 3-9. Percentage of larvae from microinjection study one with skeletal deformities related to selenium concentrations in larvae at the onset of larval development 185

Figure 3-10. Percentage of larvae from microinjection study one with edema and/or skeletal deformities related to selenium concentrations in larvae at the onset of larval development 186

Figure 3-11. Percentage of larvae from microinjection study two with edema related to selenium concentrations in larvae at the onset of larval development 187

Figure 3-12. Percentage of larvae from microinjection study two with skeletal deformities related to selenium concentrations in larvae at the onset of larval development 188

Figure 3-13. Percentage of larvae from microinjection study two with edema and/or skeletal deformities related to selenium concentrations in larvae at the onset of larval development 189

Figure 3-14. Percentage of maternally exposed and microinjected larvae with edema related to selenium concentrations in larvae at the onset of larval development 190

Figure 3-15. Percentage of maternally exposed and microinjected larvae with skeletal deformities related to selenium concentrations in larvae at the onset of larval development 191

Figure 3-16. Percentage of maternally exposed and microinjected larvae with edema and/or skeletal deformities related to selenium concentrations in larvae at the onset of larval development 192

Figure 3-17. Probabilities of mortality in larvae from female white sturgeon exposed to dietary selenium 193

Figure 3-18. Probabilities of mortality in larvae following microinjection of seleno-L-methionine 194

Figure 3-19. Probability of mortality in larvae using combined data from selenium maternal transfer and microinjection studies in white sturgeon 195

Introduction

The overarching goal of this dissertation was to elucidate the effects of selenium (Se) bioaccumulation on white sturgeon, *Acipenser transmontanus*, with an emphasis on the San Francisco Bay population. This introduction outlines the need for the studies presented here and provides additional background. Three studies are included in this dissertation. The first investigates the long-term effects of dietary Se in juvenile white sturgeon. The next study examines the mechanisms of Se maternal transfer in white sturgeon and the impacts of accumulated Se on reproduction. Finally, we investigate the effects of Se on developing white sturgeon embryos and larvae.

Selenium in San Francisco Bay-Delta

San Francisco Bay-Delta is the largest estuary in the western United States. The Delta is formed by the confluence of the Sacramento and San Joaquin Rivers. Se enters this system in local petroleum refinery wastewaters and irrigation drainage from the seleniferous western San Joaquin Valley. The fate and cycling of Se is mediated by chemical speciation, hydrological conditions and water chemistry, among other factors (Luoma and Presser 2000). In the water column, Se is assimilated and transformed into organic Se by phytoplankton (Hu *et al.* 1997; Vandermeulen and Foda 1988; Wrench 1978). The organic Se is predominately in form of protein-bound selenomethionine and selenocysteine (Fan *et al.* 2002; Wrench 1978). Organic Se in phytoplankton is consumed by filter feeders (Luoma *et al.* 1992) or recycled back to the water column upon decomposition of the plankton (Besser *et al.* 1994; Gobler *et al.* 1997; Wang and Guo 2001). In the sediment, Se undergoes microbial reduction and is transformed into

particulate elemental and organic Se (Oremland *et al.* 1990). Se in the sediment can return to the water column via pore waters (Velinsky and Cutter 1991).

Se is prevalent throughout northern San Francisco Bay and accumulates in several species found in this region. Risebrough *et al.* (1977) reported concentrations of 8 to 11 μg/g Se dry weight (dw) in transplanted bivalves in northern San Francisco Bay. Johns *et al.* (1988) reported a mean of 6 μg/g Se dw in resident bivalve *Corbicula spp.* sampled near Carquinez Strait. The Selenium Verification Study found Se concentrations of 5.13 to 7.90 μg/g dw in *Corbicula spp.* from the north Bay between 1987 and 1990 (Urquhart and Regalado 1991; White *et al.* 1988; White *et al.* 1989). The filter feeding exotic bivalve *Potamocorbula amurensis* contains an average Se level of 15 μg/g dw (Linville *et al.* 2002). This non-native species was introduced into San Francisco Bay in the mid-1980's and has since become the dominant bivalve in the bay (Carlton *et al.* 1990; Nichols *et al.* 1990) and a major food source of benthic-feeding organisms (Urquhart and Regalado 1991). The high Se level and wide distribution of *P. amurensis* is of great concern because its Se burden significantly exceeds the levels shown to cause toxicity in animals consuming bivalves. Prey items containing greater than 10 μg/g Se have been shown to induce Se toxicity in birds and fish (Adams *et al.* 1998; Coyle *et al.* 1993; Hamilton *et al.* 1990a; Heinz *et al.* 1989; Hermanutz *et al.* 1992; Hoffman *et al.* 1989; Woock *et al.* 1987). Based upon tissue burdens, the fish most vulnerable to Se contamination in San Francisco Bay are benthic feeders, including white sturgeon (Luoma and Linville 1997; Urquhart and Regalado 1991; White *et al.* 1987).

White sturgeon sampled from San Francisco Bay-Delta between 1986 and 1990 contained Se at concentrations ranging from 9 to 30 μg/g dw in liver (n= 52) and 7 to 15

μg/g dw in muscle (n= 99; Urquhart and Regalado 1991; White *et al.* 1988). Out of six sturgeon females sampled in the San Francisco Bay-Delta region in the 1990's, one contained eggs with 3 μg/g Se, four had eggs ranging from 8 to 12 μg/g Se and one female contained eggs with 29 μg/g Se (Kroll and Doroshov 1991). In 2000, white sturgeon from this region exhibited a mean liver Se concentration of 24 μg/g, with some samples reaching as high as 40 μg/g Se (n= 15; Stewart *et al.* 2004). Linares *et al.* (2004) reported a mean liver Se concentration of 9.75 μg/g in 36 sub-adult and adult (age 4 – 18) white sturgeon sampled in San Francisco Bay between 2002 and 2004. Recently, three white sturgeon captured from San Francisco Bay-Delta were found to have 7 to 20 μg/g Se in ovaries containing developing eggs (Doroshov Lab, UCD; unpublished data). The Se concentrations found in white sturgeon captured from San Francisco Bay-Delta reach levels previously linked to adverse effects in other fish (Coughlan and Velte 1989; Gillespie and Baumann 1986; Hermanutz *et al.* 1992; Lemly 1993b).

Although Se levels in white sturgeon from this region are quite high, they are also variable. Stewart *et al.* (2004) recently examined this variability and found sampling seasonality and foraging location to be likely explanations. Se levels in *P. amurensis*, a primary route of Se exposure, are strongly influenced by seasonal variability (Linville *et al.* 2002; Luoma and Presser 2000; Stewart *et al.* 2004). Se is highest in these bivalves after periods of low river outflow. Stewart *et al.* (2004) used stable isotope ratios to show that foraging location was a primary cause of high variation in white sturgeon tissue Se burden.

To mitigate the Se pollution in San Francisco Bay-Delta, the California San Francisco Bay Regional Water Quality Control Board required local oil refineries to

reduce the amount of Se in effluents discharged into the Bay; however, refinery effluent continues to be a significant source of Se in this region (Luoma and Presser 2000). The amount of Se entering the San Francisco Bay-Delta in agricultural effluents from the San Joaquin Valley may increase in coming years due to changes in water management (Luoma and Presser 2000). Therefore, Se contamination is a high priority for the management of the San Francisco Bay-Delta ecosystem.

White sturgeon in San Francisco Bay-Delta

White sturgeon is one of the two endemic sturgeons inhabiting the Pacific Northwest. Both white and green (*A. medirostris*) sturgeon have high zoological value and are important to biodiversity; but white sturgeon has a higher value as a food and game fish. Both species belong to a phylogenetically unique group of ancient ray-finned fish that has survived for over two hundred million years (Sewertzoff 1923). White sturgeon is the largest North American sturgeon species with individuals recorded at 6 meters in length and over 800 kg in weight (reviewed by Billard and Lecointre 2001; Doroshov 1985). This species is also unusually long-lived, sometimes surviving 100 years. In San Francisco Bay-Delta, white sturgeon aggregate in northern San Francisco Bay (Suisun and San Pablo Bays) and migrate to spawn in the river systems (Kohlhorst 1980; Kohlhorst *et al.* 1980).

The reproductive strategy of white sturgeon is quite different from that of modern teleosts. Sturgeon exhibit a delayed sexual maturity, with males typically maturing at age 10 to 12 years and females at 15 to 32 years (Doroshov 1985; Doroshov *et al.* 1997). White sturgeon are long-lived and reproduce repeatedly over their lifespan (Doroshov

1985). Each reproductive cycle of female white sturgeon includes at least two-years of yolk deposition in the eggs (Doroshov *et al.* 1997). Yolk deposition is dependent on the yolk precursor, vitellogenin, which is synthesized in the liver. Vitellogenin is transported by the circulatory system to developing oocytes, where it is selectively taken up by receptor-mediated endocytosis (Mommsen and Walsh 1988). In the oocyte cytoplasm, the vitellogenin is reprocessed and deposited as yolk. In contrast to other fish, sturgeon store and use their yolk in the form of intracellular crystalline platelets throughout early development (Dettlaff *et al.* 1993). Vitellogenin has also been indicated as a potential vehicle of Se maternal transport in white sturgeon. Kroll and Doroshov (1991) found high, but variable, levels of Se in isolated plasma vitellogenin and yolk proteins from six sturgeon females caught in the Sacramento River, indicating the potential risk of developmental Se toxicity in sturgeon of this region.

Spawning white sturgeon migrate up river during March through May to broadcast their eggs, which adhere to the substrate until hatching. Sturgeon embryos hatch as underdeveloped (altricial) organisms referred to as yolk sac larvae. During the first 10 days post-hatch, over eighty percent of the maternal yolk is rapidly metabolized for growth, development, and for greatly increased metabolic energy associated with pelagic swimming (Bolker 1993; Dettlaff *et al.* 1993; Doroshov *et al.* 1983). White sturgeon exhibit holoblastic development and their yolk is digested intracellularly, in contrast to the extraembryonic yolk of modern teleost fish (Buddington and Doroshov 1986; Dettlaff *et al.* 1993). Vital organs are differentiated during the yolk sac larvae period, such as the gills, liver and kidney.

After hatching, the larvae are carried down river with the current. Juvenile white sturgeon may remain in the rivers or delta for a period of years before entering the Bay (Radtke 1966). Juveniles feed on crustaceans and, to a lesser extent, bivalves (McCabe *et al.* 1993; Radtke 1966; Schreiber 1962). Adult white sturgeon spend the majority of theirs lives within San Francisco Bay (Doroshov 1985; Kohlhorst 1980; Kohlhorst *et al.* 1980). Adults in this area primarily consume mollusks and commonly feed on *P. amurensis* (McKechnie and Fenner 1971; Urquhart and Regalado 1991).

Historically, the San Francisco Bay white sturgeon population decreased dramatically in the early 1900's due to overharvesting (Doroshov 1985; Kohlhorst 1980). This population continues to be impacted by habitat destruction, reduced river flows, and overharvesting. White sturgeon populations in San Francisco Bay-Delta have been highly variable over the past five decades (Kohlhorst 1980; Kohlhorst *et al.* 1991; Schaffter and Kohlhorst 1999). Schaffter and Kohlhorst (1999) reported a paucity of smaller sturgeon caught in the late 1990's. Population surveys in 2005 indicated that the San Francisco Bay-Delta white sturgeon population was at a 50-year low, with an estimated 10,000 adult sturgeon (California Department of Fish and Game Commission 2006).

Observed decreases in the San Francisco Bay white sturgeon population have been associated with the occurrence of dry water years (Kohlhorst *et al.* 1991). This may be due to several factors, including flow rate and habitat availability. In addition, Se concentrations in sturgeon food sources significantly increase during low-flow years (Johns and Luoma 1988; Linville *et al.* 2002; Luoma and Presser 2000). The life history of white sturgeon may render them especially vulnerable to Se bioaccumulation and

maternal transport of Se to egg yolk due to a long life span, benthic feeding habits and a prolonged period of vitellogenesis and yolk deposition. Se toxicity cannot be discarded as a potential factor affecting white sturgeon reproduction and natural recruitment in San Francisco Bay-Delta.

Toxicity of selenium

Se is an essential nutrient (0.1 – 1.25 μg/g diet) for animals, humans and many micro-organisms (Diplock 1976; Gatlin and Wilson 1984a; Hilton *et al.* 1980; Mayland 1994). Se is also a significant environmental toxicant due to its efficient food web transfer (Lemly 1985; Luoma *et al.* 1992). There is a very narrow margin between nutritional and toxic concentrations of this element. Concentrations as low as 3 μg/g in diet have led to toxicity in aquatic organisms (reviewed by Hamilton 2004). Se toxicity results in reduced growth, significant histopathology in major organs, alteration of detoxification systems, promotion of oxidative damage, and mortality (e.g., Hicks *et al.* 1984; Hoffman *et al.* 1989; Lemly 1993a; Lemly 1998; Sorensen and Thomas 1988; Stanley *et al.* 1996; Woock *et al.* 1987). Se is also a well-documented developmental toxicant to fish and wildlife species consuming diets containing 13 μg/g Se or greater (e.g., Coyle *et al.* 1993; Gillespie and Baumann 1986; Hamilton *et al.* 1986; Hermanutz 1992; Hoffman *et al.* 1988a; Lemly 1993b; Ohlendorf *et al.* 1986; Woock *et al.* 1987). Common developmental defects produced by Se toxicity include severe edema (e.g., Gillespie and Baumann 1986), skeletal deformities (scoliosis, lordosis and kyphosis; e.g., Schultz and Hermanutz 1990), and craniofacial deformities (e.g., Holm *et al.* 2005). Each of these defects is likely to reduce the probability of survival in developing fishes in

the natural environment. Excess Se has led to severe teratogenesis, mortality and endangerment or extirpation of fish species residing in areas high in Se (Garrett and Inmann 1984; Hamilton 1999; Lemly 1985; Skorupa 1998). Lemly (2002) has proposed Se toxic thresholds for freshwater and anadromous fish as 8 μg/g in muscle, 12 μg/g in liver, and 10 μg/g in ovary or eggs.

Se toxicity is well documented in freshwater species (reviewed by Hamilton 2004). However, few studies have investigated Se toxicity in euryhaline and marine species. Hamilton *et al.* (1990a) investigated Se toxicity in chinook salmon (*Oncorhynchus tshawytscha*) and found that 10 μg/g of dietary Se reduced survival and 5 μg/g reduced growth. Striped bass (*Morone saxatilis*) fed Se-laden red shiners (39 μg/g) experienced complete mortality (Coughlan and Velte 1989). In the grass goby (*Zosterisessor ophiocephalus*), a marine fish, the 96hr LD_{50} for selenite via ip injection was 0.29 μg/g body weight (Tallandini *et al.* 1996). Very few studies are available on the effects of Se in white sturgeon. Recent work shows that excess Se leads to toxicity in white sturgeon. Dietary exposure of 40 μg/g seleno-L-methionine led to impaired growth in fingerling white sturgeon (25 – 30g) after two weeks (Tashjian *et al.* 2006). In the same study, the activity level of fingerling white sturgeon decreased with increasing Se exposure. A study investigating the effects of Se exposure on salinity tolerance in juvenile white sturgeon indicates a strong interactive effect of dietary Se exposure and elevated salinity on survival (Tashjian 2005). Sturgeon mortality and time to death were both positively correlated with levels of Se in the diet. To our knowledge, no studies have been published regarding the developmental toxicity of Se in white sturgeon.

The specific chemical species and route of exposure are critical in the manifestation of Se toxicity. In the natural environment, Se is found in four oxidation states, selenide (-II; Se^{-2}), elemental Se (0, Se_0), selenite (IV; SeO_3^{-2}), and selenate (VI; SeO_4^{-2}). Elemental Se is commonly found in anoxic sediments (reviewed in Frankenberger and Engberg 1998). Selenite and selenate are present as salts in the soil, or as dissolved ions in aquatic systems. Selenide is found in organic forms of Se, such as seleno-amino acids, and as dissolved ions. Organic forms of Se, particularly selenomethionine, have been shown to have greater bioavailability and toxicity than inorganic forms and are often the species to which aquatic organisms are exposed (Hamilton *et al.* 1990a; Hoffman *et al.* 1989; Luoma *et al.* 1992; Reinfelder *et al.* 1997; Wang and Lovell 1997). Additionally, the diet is the most significant route of exposure leading to Se bioaccumulation and toxicity (e.g., Coyle *et al.* 1993; Hermanutz 1992; Luoma *et al.* 1992). Much of the previous work on Se has utilized waterborne exposures (e.g., Gissel Nielsen and Gissel Nielsen 1978; Hodson *et al.* 1980; Lemly 1982). In addition, the common use of inorganic Se in both waterborne and dietary exposures underestimates environmental exposure, uptake and toxicity of Se to aquatic organisms (Fan *et al.* 2002; Wang and Lovell 1997; Woock *et al.* 1987). However, the appropriate use of selenomethionine through dietary exposure in toxicity studies is beginning to be widely recognized.

Se is readily adsorbed through the gut and metabolized in the liver and kidney through a glutathione-dependent reductive pathway (reviewed by Diplock 1976; Suzuki 2005b). Glutathione (GSH) is a tripeptide nucleophile consisting of glutamic acid, cysteine and glycine. With the aid of glutathione-S-transferase enzymes, GSH reduces

Se species to the –II oxidation state (selenide). As a result, GSH becomes oxidized and must be reduced by NADPH-dependent glutathione reductase before it can react with another Se compound. The serial reduction of selenate and selenite by GSH produces the intermediary metabolite selenodiglutathione (GSSeSG), which is further reduced to glutathione selenopersulfide (GSSeH). Glutathione selenopersulfide is reduced to hydrogen selenide (H_2Se), which may undergo autooxidation back to glutathione selenopersulfide (Anundi *et al.* 1984). Seleno-amino acids are transformed to selenide by methylation (Spallholz *et al.* 2004) or cleavage of Se through lyase functions (Esaki *et al.* 1982; Okuno *et al.* 2005a; Okuno *et al.* 2005b). Selenide can then undergo various reactions that could lead to toxicity or excretion.

Se excretion is mediated through methylation by methyltransferase and the cofactor S-adenosylmethionine (SAM); however, many methylated metabolites are also toxic (reviewed by Diplock 1976; Francesconi and Pannier 2004). Exposure to excess Se leads to selenosugars and trimethylselenonium ion in the urine (Byard 1969; Kobayashi *et al.* 2002). At very high Se levels, the step from di- to tri-methlyation becomes limiting and dimethylselenide is excreted through respiration (McConnell and Portman 1952).

There are several potential modes of action in Se toxicity. Metabolic intermediates of Se are thought to be a significant cause of Se toxicity. Methylselenol, an intermediate metabolite of both selenite and selenomethionine, can lead to superoxide production resulting in cellular disruption (Okuno *et al.* 2005b; Seko *et al.* 1989; Yan and Spallholz 1991). In fact, the production of reactive oxygen species may be responsible, in part, for the destruction of cancer cells observed with high-Se treatment (reviewed by Drake 2006; Spallholz 1994). The metabolic intermediate selenodiglutathione has been

indicated in the inhibition of protein synthesis (Vernie and Van Leewenhoekhuis 1987), the reduction of cellular growth (Wu *et al.* 1995), and the induction of apoptosis (Lanfear *et al.* 1994). The metabolite hydrogen selenide is also extremely toxic (reviewed by Spallholz and Hoffman 2002).

Additionally, metabolism of excess Se can alter natural detoxification systems by depleting reduced GSH or the methylation co-factor SAM (Anderson and Moxon 1942; Hoffman and Heinz 1988; Hoffman 1977). Reduced GSH, along with glutathione peroxidase, is crucial in detoxifying peroxides and halting lipid peroxidation. With the aid of glutathione transferase, reduced GSH actively binds electrophilic toxins and prevents them from binding to macromolecules (proteins, DNA, enzymes, etc.). Methylation is also a common detoxification pathway. Depression of these essential detoxification mechanisms leaves organisms vulnerable to additional toxic insults.

Se can also be incorporated into biological molecules in place of sulfur because the two elements have similar chemical properties (Diplock 1976; Stadtman 1974). Se is incorporated into proteins as a result of genetic translation (reviewed by Gromer *et al.* 2005; Suzuki 2005b). A specific genetic codon prescribes the incorporation of selenocysteine into proteins; however, selenomethionine is indiscriminately substituted for the methionine codon in genetic translation. The amount of selenomethionine that is indiscriminately incorporated into proteins increases with increasing availability of selenomethionine in tissues (Schrauzer 2000). Similarly, when increased Se is available compared to sulfur, selenocysteine can substitute for cysteine in protein synthesis (Kramer and Ames 1988; Müller *et al.* 1994). However, Se and sulfur bonds may have different strength and function in biological molecules (Reddy and Massaro 1983), thus

some Se-substituted compounds may become unstable or dysfunctional (Lemly 1998; Stadtman 1974).

Research aims

The studies presented here were designed to identify potential Se toxicity in white sturgeon, at exposures relevant to the San Francisco Bay-Delta population. Understanding the effects of Se on white sturgeon is a critical link between studying the extent of Se contamination in biota and regulating Se loading in this region. The recruitment success of fishes is not dependent on a singular factor, but rather a synergistic interaction of several factors (Bennett and Moyle 1996). Simply addressing high-profile environmental factors, such as habitat loss, may not adequately protect the white sturgeon population of San Francisco Bay-Delta unless the risk of Se toxicity is fully understood and mitigated.

Chapter 1. Toxicity of foodborne selenium to juvenile white sturgeon, with an emphasis on histopathology of the liver

Introduction

In small quantities (0.1 – 1.25 μg/g diet) selenium (Se) is an essential nutrient for animals, humans and many micro-organisms (Diplock *et al.* 1976; Gatlin and Wilson 1984b; Hilton *et al.* 1980; Mayland 1994). In slightly greater quantities (as little as 3 μg/g Se in diet) this element is a potent environmental toxicant that threatens higher trophic level species because of its efficient food web transfer (Hamilton 2004; Lemly 1985; Lemly 2002; Luoma *et al.* 1992; Stewart *et al.* 2004). Environmental exposure to Se has led to population-level effects in birds and fish residing in areas receiving wastewaters from the combustion of fossil fuels (Garrett and Inmann 1984; Lemly 1985) or drainage from seleniferous land, including both agricultural and natural drainage (Hamilton 1999; Presser and Ohlendorf 1987; Skorupa 1998). Se toxicity is well studied in warm, freshwater systems (Hamilton 2004), but its effects are largely unknown in estuarine systems (Luoma and Presser 2000; Skorupa 1998).

San Francisco Bay-Delta is the largest estuary in the western United States. The Delta is formed by the confluence of the Sacramento and San Joaquin Rivers. Se enters this system in petroleum refinery wastewaters and irrigation drainage from the seleniferous western San Joaquin Valley. White sturgeon, *Acipenser transmontanus*, resides in San Francisco Bay (Kohlhorst *et al.* 1991) and is exposed to high levels of Se through their diet. This is evidenced by chemical detection of high tissue Se levels in common prey of white sturgeon (Linville *et al.* 2002; Stewart *et al.* 2004; White *et al.* 1988). In addition, sturgeon muscle, liver and eggs show significant levels of Se,

indicating that diet is a predominant route of exposure to this element (Kroll and Doroshov 1991; Stewart *et al.* 2004; Urquhart and Regalado 1991; White *et al.* 1989).

Common San Francisco Bay bivalves were shown to contain 3 to 9 μg/g Se (all values listed as dry weight) in the 1980's (Johns and Luoma 1988; Urquhart and Regalado 1991; White *et al.* 1988; White *et al.* 1989). However an invasive bivalve, *Potamocorbula amurensis,* introduced into San Francisco Bay in the mid-1980's accumulates significantly higher levels of Se, an average of 15 μg/g (Linville *et al.* 2002; Luoma and Presser 2000; Stewart *et al.* 2004). Since being introduced, *P. amurensis* has become the dominant bivalve in the bay (Carlton *et al.* 1990; Nichols *et al.* 1990) and a major current food source of adult white sturgeon (Stewart *et al.* 2004; Urquhart and Regalado 1991). Juvenile white sturgeon also consume bivalves, although not predominantly (McCabe *et al.* 1993; Radtke 1966; Schreiber 1962). The high Se level and wide distribution of *P. amurensis* is of great concern because consumption of prey containing greater than 10 μg/g Se induces Se toxicity in birds and fish (Coyle *et al.* 1993; Hamilton 2004; Hamilton and Buhl 1990; Hamilton *et al.* 1990b; Hermanutz *et al.* 1992; Hoffman *et al.* 1989; Woock *et al.* 1987).

White sturgeon aggregate in north San Francisco Bay (Suisun and San Pablo Bays) and migrate to spawn in the river systems, predominately the Sacramento River (Kohlhorst 1980; Kohlhorst *et al.* 1980). Juveniles migrate back to the Bay-Delta, but may remain in the lower Sacramento River for several years (Radtke 1966). White sturgeon has a very high value as a food and game fish and is important to biodiversity in the Pacific Northwest. Two endemic species of the Pacific Northwest, white and green (*Acipenser medirostris*) sturgeons, belong to a phylogenetically ancient group of ray-

finned fish that has survived for over two hundred million years, but has experienced rapid decline over the past century (Birstein 1993).

White sturgeon sampled from San Francisco Bay between 1986 and 1990 contained an average of 12.57 μg/g Se in muscle (± 9.72 standard deviation; range of 1.46 – 50 μg/g Se; n= 99) and an average of 26.55 μg/g Se in liver (± 21.76 standard deviation; range of 3.24 – 79.42 μg/g Se; n=54; Urquhart and Regalado 1991; White *et al.* 1987; White *et al.* 1988; White *et al.* 1989). In 2000, white sturgeon from this region had a mean liver Se concentration of 24 μg/g with some samples reaching as high as 40 μg/g Se (n = 15; Stewart *et al.* 2004). These concentrations exceed concentrations previously linked to adverse effects in other fish (Coughlan and Velte 1989; Hermanutz *et al.* 1992).

Elevated Se affects growth, condition factor, hepatosomatic index, and survival in several fish species (Hamilton *et al.* 1990b; Hilton *et al.* 1980; Sorensen and Bauer 1984; Sorensen *et al.* 1984; Woock *et al.* 1987). However the current literature reflects a lack of consistency in these effects, which may be linked to differences between the species, life-stages, and experimental conditions (Hamilton 2004). Exposure to elevated Se concentrations also causes histopathological alterations in several fish species (Coughlan and Velte 1989; Ellis *et al.* 1937; Hicks *et al.* 1984; Lemly 1993a; Sorensen 1988; Sorensen *et al.* 1984). Se can result in damaged or necrotic liver cells; and, thereby alter the structural framework of the organ in survivors (Coughlan and Velte 1989; Ellis *et al.* 1937; Sorensen *et al.* 1982a; Sorensen *et al.* 1983a; Sorensen *et al.* 1983b; Sorensen *et al.* 1984). In the kidney, excessive Se concentrations affect glomerular and tubular epithelial cells and can lead to disease states such as proliferative glomerulonephritis,

nephrocalcinosis and uremia (Coughlan and Velte 1989; Hicks *et al.* 1984; Sorensen *et al.* 1983a; Sorensen *et al.* 1982b). Gills of Se-exposed fish exhibit swollen and vacuolated secondary lamellae, which can adversely affect respiration and could potentially lead to death (Lemly 1993a; Sorensen *et al.* 1982a; Sorensen *et al.* 1984). Se is also a potent reproductive and developmental toxin when transferred from the adult female to the yolk of their eggs (Gillespie and Baumann 1986; Lemly 1993b).

Due to a paucity of information regarding the effects of Se on sturgeon, this study was initiated to determine the susceptibility of juvenile white sturgeon to chronic Se exposure via diet. We reared sturgeon juveniles using three foodborne Se (as selenized yeast) levels and a control diet with the required low Se level for essential nutrition. Selenized yeast, predominately in the form of selenomethionine (Polatajko *et al.* 2005), was used as a surrogate for organic Se present in the aquatic environment. Organic forms of Se, particularly selenomethionine, have been shown to have greater bioavailability and toxicity than inorganic forms and are often the species to which aquatic organisms are exposed (Hamilton *et al.* 1990b; Hoffman *et al.* 1989; Luoma *et al.* 1992; Reinfelder *et al.* 1997; Wang and Lovell 1997). Following six months of rearing, the juveniles were sacrificed and tissue Se concentrations were measured. In addition, growth, and histopathological and morphological alterations were assessed. Our hypothesis was that dietary Se at environmentally relevant levels would be accumulated in sturgeon tissues, inhibit growth, and induce pathological changes.

Materials and Methods

Source of Animals

White sturgeon juveniles (6 weeks post hatch, mean weight 350 mg) were obtained from Stolt Sea Farm, California in May 2000. They originated from the same fertilization mixture composed of gametes from three female and six male hatchery-bred white sturgeon. Larvae were transported to University of California, Davis (UCD) and reared to 38 weeks post-hatch following standard hatchery protocols (Conte *et al.* 1988) and monitored for health. During early rearing, a portion of the juveniles were euthanized due to an outbreak of white sturgeon iridovirus (Georgiadis *et al.* 2001). However, by 25 weeks post-hatch there were no signs of fish illness and the fish exhibited normal growth and survival until 47 weeks post-hatch, prior to initiating the study.

Experimental Design

Hatchery reared juveniles (47 weeks old, average weight 575 g) were held in twelve, 5-foot diameter, outdoor flow-through tanks at the Center for Aquatic Biology and Aquaculture, UCD. The experiment consisted of four dietary treatments: control (1.04 ± 0.07 μg/g Se), low (20.05 ± 1.10 μg/g Se), medium (35.61 ± 3.83 μg/g Se) and high (52.54 ± 2.32 μg/g Se; mean ± standard deviation; Table 1-1). Each dietary treatment was replicated in three randomly assigned tanks, with 10 fish per tank and 30 fish per treatment.

Initiation and maintenance

At 38 weeks post-hatch, 144 randomly selected fish were anesthetized (200 mg/L MS-222) measured, weighed and randomly distributed into the experimental tanks. Each tank initially contained 12 fish with mean weight of 465 g, fork length of 39.3 cm and condition factor of 0.76 (100 x W/L^3). There were no statistical differences among treatment tanks (ANOVA; $p > 0.82$). At 46 weeks post hatch, two fish from each tank were sacrificed to provide an initial Se measurement using techniques described below. Three days later the remaining 120 fish were gradually transitioned onto respective experimental diets over one week. All juveniles were on full experimental diets by 47 weeks-post hatch. The experimental tanks received well water at an average flow rate of 168 L/min. The temperature in the tanks averaged 18.6 °C. The dissolved oxygen and percent air saturation averaged 9 mg/L and 95% respectively. The fish were observed daily for mortality and morbidity. Moribund fish were euthanized using MS-222 overdose. The tanks were cleaned weekly, with minimum disturbance to the fish. Following 23 weeks of dietary Se exposure, the fish were measured for fork length and wet weight, and were necropsied for tissue analyses.

Experimental Diet

Sturgeon were fed a casein-based, purified pelleted diet (Table 1-2), specifically formulated for sturgeon and with a satisfactory history for rearing hatchery sturgeon stocks for longer than two years with consistently good growth and free from any abnormal signs (Hung *et al.* 1987). Optimal feeding rates and nutrient requirements of white sturgeon are known (Hung 1991; Hung and Deng 2002). Dietary ingredients were

mixed and then pelleted as described by Hung and Lutes (1987). Four diets were formulated with increasing levels of Se (Table 1-1). Se was added to the diet as selenized yeast (Selenomax®, Ambi Inc.). Sturgeon were continuously fed the experimental diet, using a 24 hour belt feeder, at a rate of 1.2% body weight per day.

Sampling

After 23 weeks of dietary Se exposure, sturgeon were sampled over a three-day period. Fish were stunned by a quick blow to the head, weighed and measured. Blood was collected from the caudal vein using a 10 ml heparinized Vacutainer® and centrifuged (4000 x g, 10 min) to separate the plasma, which was flash frozen in liquid nitrogen for Se analysis. Fish were then euthanized by MS-222 overdose and the spinal cord was cut before proceeding with the necropsy. Dissected liver and gonads were weighed for respective somatic indices. Tissue pieces from gill, gonad, liver and posterior kidney were fixed in 10 volumes of 10% phosphate-buffered formalin for histopathological analysis as described below. In addition, samples of liver and muscle were frozen on dry ice and stored at -80 °C for Se analysis.

Selenium Analysis

Frozen tissue samples were lyophilized and weighed. Muscle samples were homogenized using a Wiley Mill. Liver samples were flash-frozen in liquid nitrogen and then pulverized with a mallet. The entire sample of lyophilized plasma was analyzed. Approximately 0.25 g of lyophilized tissue was digested using a nitric/sulfuric/perchloric acid mixture heated to 230 °C over two days. Se in the digested solution was reduced to

selenite by the addition of 6M HCl heated to 90 °C for 1 hour. The reduced samples were diluted to 25 ml with 4 M HCl and stored at 4 °C until spectroscopy analysis (less than 10 days).

Total Se was analyzed as selenite using Hydride-Generation Atomic Absorption with an EDL Se lamp (PerkinElmer AAnalyst 300 Atomic Adsorption Spectrometer MHS-15). The instrument had a minimum detection limit of 1 ng Se and was calibrated in the linear region, from 20 to 100 ng Se, using a sodium selenite standard solution. Quality control included spikes, duplicates, certified reference materials (NRC DORM-2, DOLT-2, DOLT-3) and validation by an external laboratory (California Animal Health and Food Safety Laboratory at UCD). All quality control measurements agreed with the target concentration within 15 percent. All blanks were less than 1 ng/ml Se. All data is reported as dry weight. Moisture content was determined as 58.3 ± 6.59 percent in liver and 77.6 ± 1.21 percent in muscle (mean percent moisture ± standard deviation, n=117). Plasma contained 95.9 ± 0.41 percent moisture and 42.02 ± 5.7 mg of dry weight per milliliter.

Gross Morphology

Condition factor was measured as (weight (g)/fork Length^3 (cm)) * 100. Specific growth rate (SGR) was determined for each tank using the formula: SGR = 100 * ($\ln Weight_{final} - \ln Weight_{initial}$)/time (days). Organ to body weight relationships were analyzed by regressing log organ weights by log body weights. Body weight to length relationships were analyzed by regressing log body weights against log fork lengths. Log transformations were used to account for allometric growth.

Histological Analyses

At necropsy, following euthanasia, the ventral abdominal wall was surgically opened and tissues were rapidly removed for placement in fixative. Liver (1-3 mm thick piece of the left lateral margin), kidney (similarly sized pieces from rostral and caudal portions), and gonad were removed. Next, the second gill arch was identified and removed after transaction at dorsal and ventral midlines. Finally, a wedge of skeletal muscle from the flank with skin attached was removed and, using a fresh, clean razor blade, a 1-3 mm piece of muscle was trimmed from this sample. Tissues were immediately immersed in 10% neutral buffered saline and stored under refrigeration in individually labeled containers at a ratio of 10 volumes of fixative to 1 volume of tissue. After completion of fixation, solution was decanted into appropriately labeled storage chambers and each container was filled with 0.1 molar phosphate buffer containing 6% sucrose and stored under refrigeration (2 – 4 °C) until time of processing. At the initiation of processing, fixed tissues were trimmed, selecting the outermost (1-2 mm thick) portions to ensure fixative penetration, dehydrated in graded ethanol solutions, cleared, and embedded in paraffin. Sections (6-8 microns thickness) were made using a rotary microtome, and were attached to glass slides coded for each individual and treatment, stained with hematoxylin and eosin, coverslipped and viewed using a Nikon Eclipse E600 microscope with CFI plan fluor objectives. Images were acquired using a Nikon DXM1200 Digital Still Camera and EclipseNet ® Nikon E600 software, individually labeled with original magnification recorded. Images were stored as TIFF files in Adobe Photoshop 6.0.

Statistical Analysis

Se accumulation was compared across tissues and treatments. Because no suitable transformations to normality could be found for this data, the non-parametric Rank F-Test was used (Neter *et al.* 1996). To compare mean Se values, a least squares means Students t-test with Bonferroni-adjustment was applied to the ranked data (experiment-wise $\alpha = 0.05$; Bonferroni adjusted comparison-wise $\alpha = 0.001666$). Morphologic measurements were compared across treatments using ANOVA and ANCOVA, or Rank F-test when the assumptions of the former models could not be met (Neter *et al.* 1996). Log_{10} transformations were used in regressions of organ to body weight to account for allometric growth. Histological survey of liver, kidney, and gill was made and alterations from control anatomy were imaged, ranked as to treatment and recorded. For areal analysis of melanomacrophage centers, random, non-overlapping fields of liver were selected and the number and area of melanomacrophage centers were recorded. Comparisons of the presence of histological lesions in gills across treatment levels were made using two-way contingency tables. Statistical software packages JMP and SAS (SAS Institute Inc.) were used for all computations.

Results

Selenium Bioaccumulation

Se bioaccumulation was compared across tissues and treatments (Table 1-3). Se in each tissue increased with increasing Se in the diet (Rank F-Test, $p = .0334$; adjusted multiple comparisons, $\alpha = 0.05$; Figure 1-1). In the control treatment (*ca.* 1 μg/g dietary Se) Se accumulated more in blood than in muscle, but Se in liver was not different from

either blood or muscle. There were no differences between Se levels in tissue types in both the low and medium treatments (*ca.* 20 & 36 μg/g dietary Se, respectively). In the high treatment (*ca.* 53 μg/g dietary Se), Se accumulated more in liver than in muscle, but Se in blood was not different from either liver or muscle. Within treatment groups, there were no significant relationships between Se accumulation and condition factors (Regression; $p > 0.05$). Before initiating the experiment, 24 juvenile white sturgeon were sacrificed to provide a baseline measurement of Se. Prior to the dietary exposure, the sturgeon had low levels of Se in muscle (1.4 ± 0.20 μg/g, n=17), liver (1.89 ± 0.69 μg/g, n=10) and blood (3.66 ± 0.83 μg/g, n=10; mean Se ± standard deviation).

Morphologic Characteristics

Several whole body morphologic characteristics were compared across Se treatments. We found no differences between treatments in condition factor, fork length, body weight, or specific growth rate (ANOVA, $p > 0.075$; Tables 1-4 & 1-5). Similarly, gonadosomatic, hepatosomatic and weight to length regression slopes were not different between treatments (ANCOVA, $p = 0.08 – 0.3$; Table 1-4).

Survival

Mortality was not significant in this experiment. One mortality occurred in a high treatment tank just prior to initiating dietary Se exposures. A moribund fish was euthanized (MS-222 overdose) in a high treatment tank after it was found upside down and motionless during the second month of dietary exposure. One fish from a low treatment tank was euthanized (MS-222 overdose) in the fourth month of dietary

exposure because it was unable to swim and was emaciated. These fish were not sampled for Se or histopathology. No other mortalities were observed during the experiment.

Histopathology

Pathological changes were observed in liver, gill arch and posterior kidney of treatment fishes. Histology of gonads revealed mostly adipose tissues with very limited vasculature. No actual gonadal tissue was visible, which is typical in white sturgeon at this age class (Doroshov *et al.* 1997). The majority of pathological changes were observed in the liver.

Liver

Control liver morphology

In control fish (*ca.* 1 μg/g dietary Se), hepatocytes were the major cell type and occupied the most volume in microscopic fields (Figure 1-2). When viewed in longitudinal arrangement, two rows of hepatocytes commonly intervened between adjacent profiles of the microvasculature (sinusoids). This pattern of hepatocyte arrangement is the tubule, common as the adult phenotype in lower vertebrates including fishes (Hinton and Couch 1998). Hepatocytes contained a single nucleus but not all planes of section through hepatocytes revealed a nuclear profile due to the much larger component volume of the cytoplasm (Figure 1-2). With hematoxylin and eosin (H&E) stained preparations of paraffin-embedded liver, the nuclear margins were basophilic (dark gray) and a single nucleolus was seen (Figures 1-2 & 1-3). The cytoplasm stained lightly eosinophilic (pink). Within the cytoplasm, vacuolar profiles likely denoted lipid

that was removed during tissue processing (alcoholic dehydration). Other regions of cytoplasm stained more diffusely (gray-pink) but were not comprised of lipid profiles (Figures 1-2 & 1-3). These were interpreted as areas of glycogen storage. Evidence for hepatocyte polarity was shown (Figures 1-2 & 1-3). Hepatocyte nuclei were located in basal regions of cells (i.e., closer to hepatic sinusoids) while apical cytoplasm was only lightly stained (Figures 1-2 & 1-3) and was directed toward center of hepatic tubules (Figures 1-2 – 1-4).

The parenchyma was comprised of tubular arrays of hepatocytes and, in certain regions, individual tubules curved and abutted against adjacent tubules resulting in what appeared as sheets of cells (Figures 1-2 – 1-4). Sinusoids, the hepatic microvasculature, were easily denoted by the presence of erythrocytes whose cytoplasm stained red and contrasted with the single darkly stained nucleus (Figures 1-2 & 1-3). The luminal surfaces of sinusoidal endothelial cells were concave and nuclei often showed similar shape. These cells delimited the sinusoids and were separated from adjacent hepatocytes by a perisinusoidal space containing perisinusoidal macrophages. Within this space, small cells with high nuclear to cytoplasmic ratio and containing a single lipid droplet were seen (not shown in figures). Respectively, these cells are the fat-storing cells of Ito (Hampton *et al.* 1985; Hinton *et al.* 1987) which may become activated and assume fibroblastic properties (Hampton *et al.* 1985).

Stromal features included venules (Figures 1-2 & 1-5), arterioles, veins (Figure 1-3), small muscular arteries, bile ductules and small diameter bile ducts. Because all livers were sampled at the left margin, larger vascular and biliary structures more closely associated with the hepatic hilus (near midline of abdominal cavity) were not included.

Biliary epithelial cells frequently accompanied venules (Figure 1-5). The latter were interpreted as branches of the hepatic portal vein, the afferent venous supply to the liver. In controls these sites also contained focal aggregates of melanomacrophages (Figure 1-6). Eosinophilic granular leukocytes were frequently seen near small bile ducts (Figure 1-5). Larger diameter veins were often associated with bile ductules and hepatic arterioles (Figure 1-3), resembling mammalian portal triads (Hinton and Couch 1998). However, foci of mononuclear cell infiltration were common in these sites and melanomacrophage aggregates near large veins were usually of greater diameter than those associated with venules. In addition to accompanying veins, melanomacrophage aggregates were frequently associated with arteries, often surrounding muscular layers and periadventitia of these vessels (Figures 1-5 & 1-6). Eosinophilic granular leukocytes were found near arteries, arterioles; and in inflammatory foci they surrounded small bile ducts (Figures 1-5 & 1-7).

Low Selenium dietary group

Mild expansion of number and area of melanomacrophage aggregates was observed in the low Se treatment (*ca.* 20 μg/g dietary Se). There was a 30% incidence of tubuloform hepatocellular swelling (Figures 1-8 & 1-9). Bile duct proliferation, especially involving transitional biliary epithelial cells, was seen (Figures 1-10 & 1-11). This occurred in approximately 70% of individuals. In addition, apparent biliary stasis led to marked increase in the volume of the canalicular network. Canaliculi are the initial portions of the intrahepatic biliary system receiving bile made by hepatocytes. Normally, in paraffin-embedded material these passageways are not seen; however, in this group the

condition was seen in each individual. Bile stasis is a hallmark feature of hepatotoxicity and criteria for this condition fit those of the medical diagnosis (Lee 1996-2005). Limited formation and subsequent excretion of bile, due to stasis, results in liver necrosis and animal death if prolonged and severe (Beers 2003; Lee 1996-2005). Perivascular inflammation was enhanced over control morphology in 40% of the individuals of this group.

Medium selenium dietary group

Observed changes in the medium treatment (*ca.* 36 μg/g dietary Se) included moderate expansion of melanomacrophage aggregates in area and in number; increased perivascular inflammation; focal hepatocellular necrosis with accumulation of discreet eosinophilic cytoplasmic inclusions; biliary canalicular distension and biliary epithelial cell (ductular and small ducts) proliferation (Figures 1-12 & 1-13).

High selenium dietary group

Alterations in the high treatment (*ca.* 53 μg/g dietary Se) involved hepatocytes (fatty change, biliary stasis of canalicular network, necrosis, reduction in hepatocyte volume in cells of portal zones, and presence of Councilman bodies which are evidence of hepatocellular apoptosis), mononuclear leukocytes (foci of inflammation), macrophages (melanomacrophage aggregation), and biliary epithelial cells (proliferation of ductules and small ducts; Figures 1-14 & 1-15). Maximum melanomacrophage aggregation response occurred in this group (Figure 1-14). There was evidence of areas of parenchyma being divided by septae of proliferating bile ductules. In addition, a

mosaic of response was seen in some individuals indicating that two functionally distinct populations of hepatocytes were present. First, vacuolated hepatocytes (suspected but unconfirmed lipid change) occurred in distinct zones and these zones were lacking in melanomacrophage aggregates. The next zone was one in which hepatocytes were shrunken, stained more intensely, and were likely producing apoptotic fragments which were then phagocytosed by adjacent cells producing bright red Councilman bodies. These regions contained the melanomacrophage aggregates. As in other Se concentration groups, the canalicular network was distended; and this, along with distension of bile ductular and small ductal lumina, suggests an inability to transport bile from the liver. This constitutes a major form of toxicity to the liver and may account for some of the cytotoxic effects following Se exposure.

That small bile duct proliferation may be seen with other hepatic conditions was suggested by the finding of duct proliferation and bile stasis in one control fish. The high amplitude swelling of some tubules (seen in one control and in several individuals of the medium and high Se groups) suggests a lack of volume regulation and is a form of acute hepatic tubular necrosis. This condition affected groups of individual hepatocytes of individual tubules. The consistent canalicular distension, seen only in Se exposed sturgeon constitutes proof that these animals are responding to their dietary exposure.

Gill

Gills were analyzed for the occurrences of four prominent lesion types: gill dysplasia, the misshapen secondary lamellae and sometimes bent primary lamellae, which can occur with exposure of young organisms to Se; fusion of secondary lamellae,

which results in reduced surface area for gaseous exchange; inflammation, representing a response to injury; and torsion of lamellae, where the lamellae are twisted along the long axis. Contingency tables were used to analyze the presence or absence of each lesion type in control and treatment fish. Significant differences were found for the presence of the fusion of secondary lamellae and inflammation across treatments ($p = 0.024$ and 0.041, respectively; Figure 1-16). These effects were observed more frequently in the lowest treatment group (*ca.* 20 μg/g dietary Se). No statistically significant differences were found for the presence of gill dysplasia or torsion of lamellae between treatment groups ($p = 0.25$ and 0.146, respectively).

Kidney

Kidneys of juvenile sturgeon of this study did not appear affected by Se exposure. All kidneys of juvenile groups showed signs of tubulogenesis. However, since the basophilic tubules with numerous mitotic cells and the basophilic glomeruli were seen in the control, low, medium and high Se exposure groups, this is interesting but likely not a toxic response. In addition, the tubulo/glomerulo-genesis appeared to be localized in the periphery of all kidneys, which indicates that the stem or progenitor cells for this generation are located in the same topographic site in the organ.

Eosinophilic material (putative protein) was observed in the glomerular space. This was accompanied by eosinophilic bodies in the cytoplasm of proximal tubular epithelial cells, which was interpreted as epithelial cell protein uptake as an effort to conserve protein. However this change appeared not only in animals of the experimental groups but also in the control. Despite careful analysis of glomerular space, renal

proximal tubules, intertubular structures, and generation/regeneration of tubules and glomeruli, no features of toxic alterations were seen in kidneys of exposed fish. Dietary Se did not appear to cause renal toxicity under the conditions of this experiment.

Discussion

The pattern of bioaccumulation observed in this experiment concurs with several other studies showing the efficient uptake of Se in fish exposed to increasing dietary concentrations (Cleveland *et al.* 1993; Crane *et al.* 1992; Hamilton *et al.* 1990b; Hamilton *et al.* 2002b; Hilton *et al.* 1980). The greater Se accumulation in the liver observed in the highest treatment diet (*ca.* 53 μg/g dietary Se) is expected because this organ is very active in Se metabolism and detoxification (Diplock 1976; Figure 1-1). High variation was observed in liver Se levels for each of the three treatment groups (*ca.* 20 – 53 μg/g dietary Se). One possible explanation for this variation is that varying liver lipid content between individuals could have biased the quantification of Se (expressed per gram total dried liver tissue). Sturgeon liver has been observed to contain high lipid content, especially in cultured fish (Doroshov 2004; Hung 2004; USACE 2003). Se is not typically found in lipids; and, the weight of this component may have biased the Se determination in high lipid samples (Cappon and Smith 1982; Lemly 1982). It is also possible that fish consuming greater amounts of the experimental Se diet would accumulate more Se in their livers compared to those consuming lesser amounts of the same diet. However, there was no relationship between Se accumulation and condition factor in any treatment group.

Se in all tissues of fish receiving low, medium and high experimental diets (*ca.* 20 – 53 μg/g dietary Se) reached levels that have been associated with toxicity in other fish species (Garrett and Inmann 1984; Hermanutz *et al.* 1992; Hilton *et al.* 1980; Hodson *et al.* 1980; Sorensen and Bauer 1984). Lemly (2002) proposed thresholds of Se toxic effects for freshwater and anadromous fish at 8 μg/g in muscle and 12 μg/g in liver. Mean liver and muscle Se concentrations surpassed these thresholds in each of the three treatment levels of the current study (Figure 1-1).

Se-induced toxicity in teleosts has led to reduced juvenile growth, changes in condition factor, changes in organ to body weight ratios, tissue damage in gills, liver, kidney, blood, heart and gonads, and mortality (Cleveland *et al.* 1993; Crane *et al.* 1992; Hamilton *et al.* 1990b; Hermanutz *et al.* 1992; Hilton *et al.* 1980; Lemly 1993a; Lemly 1993b; Lemly 1998; Sorensen 1988; Sorensen 1986; Sorensen *et al.* 1984; Woock *et al.* 1987). In the current study, we found no significant differences between treatments in survival, specific growth rate, condition factor, fork length, or body weight of juvenile white sturgeon, athough there was an apparent trend of decrease in length, weight, and growth rate in the high Se treatment (Tables 1-4 & 1-5). In a recent study, dietary exposure of 40 μg/g seleno-L-methionine led to impaired growth in fingerling-stage (0+) white sturgeon (25 – 30g) after two weeks (Tashjian *et al.* 2006). Our older juveniles (1+) may not be as susceptible to Se-induced growth effects and mortality. However, one study found reduced survival and growth in adult bluegills *(Lepomis macrochirus)* exposed to 10 and 30 μg/L waterborne Se for the duration of one year (Hermanutz *et al.* 1992). The influence of age on growth impacts in Se-exposed fish remains unclear.

Similarly, there were no significant differences in hepatosomatic, gonadosomatic or weight to length regressions during the course of this study (ANCOVA, p 0.08 – 0.3; Table 1-4). These findings contradict previous work with Se in modern teleosts. Sorensen *et al.* (1984) found increased liver weights in green sunfish (*Lepomis cyanellus*) collected from a high Se area compared to those collected from a reference area (based on wet weights). Significant effects on body size have also been documented with exposures to elevated Se. Hilton *et al.* (1980) observed food avoidance and feeding inefficiency (high feed: gain ratio) in rainbow trout (*Salmo gairdneri*) fed a diet containing 15 μg/g Se as sodium selenite. The absence of mortality and gross morphological effects in the current study may be due to differences in phylogeny (Chondrostei vs. Teleostei lineages), age, Se species, exposure route and duration of exposures.

Histopathological analysis of treatment liver revealed significant cholestasis with compensatory changes in biliary epithelium, and expansion of melanomacrophage aggregates in all treatment exposures (*ca.* 20, 36, & 53 μg/g dietary Se). This indicates that Se interferes with the removal of bile from the liver, which in turn causes the expansion of canaliculi, cholangioles (smallest bile ductules) and proliferation of ductules and ducts in the sturgeon liver. Yellow pigment was seen in the cytoplasm of some hepatocytes, indicating cholestasis. The disruption of bile flow (cholestasis or bile stasis) is a potentially fatal condition. Normal bile flow depends on a complex transport system. Bile acid synthesis and secretion are vital to several critical functions including the elimination of endogenous toxins, xenobiotics and their metabolites, and the assimilation of lipid soluble dietary nutrients (e.g., vitamin A, K, E and triglycerides; Groothuis and

Meijer 1996; Nathanson and Boyer 1991). Bile acids that are accumulated in liver during cholestasis damage mitochondria, generate oxidative stress, and can lead to cell death (Palmeira and Rolo 2004; Rolo *et al.* 2004; Sokol *et al.* 1995).

Cholestasis is infrequently reported with Se toxicity and is not typically reported for Se-exposed fish. However, this condition was associated with Se toxicity in birds and some mammals. Mallards (*Anas platyrhynchos*) fed a diet containing 32 μg/g Se for 14 weeks incurred bile duct hyperplasia accompanied by elevated plasma alkaline phosphatase activity and lipid peroxidation (Hoffman *et al.* 1991). Mallards fed 60 μg/g Se for 22 to 50 days exhibited bile duct hyperplasia and hepatic necrosis (O'Toole and Raisbeck 1997). Adult birds from Kesterson Reservoir, CA with mean liver Se concentrations of 94.4 μg/g (dry weight) showed bile duct hyperplasia and hepatic lesions (Ohlendorf *et al.* 1988). Buffalo calves (*Bubalus bubalis*) fed Se-enriched wheat straw (8.54 μg/g Se) for 14 days also showed markedly increased alkaline phosphatase activity levels and enlarged gall bladders (Singh *et al.* 2002). Pigs exposed to 24.5 to 49 μg/g Se via diet for up to 80 days showed bile deposition and necrosis in the liver (Miller and Schoening 1938).

While cholestasis is well recognized in rodents and mammals, this condition is only beginning to be understood in fish species (Cai *et al.* 2001; Kirby *et al.* 1995; Lorent *et al.* 2004). Cholestasis can result from genetic defects, toxins, disease, inflammation and oxidative stress (Trauner *et al.* 2005). Reduced expression and function of bile transport proteins are important factors in the development of cholestasis (Elferink 2003; Trauner *et al.* 1998). Toxins can effect the formation and secretion of bile, presumably by affecting transport protein expression and function (Trauner *et al.* 1998). Schmitt and

colleagues (2000) demonstrated that oxidative stress could lead to cholestasis by reducing the number of functional transporters in the bile canalicular membrane. These authors concluded that severe glutathione depletion could ultimately lead to cholestasis. It is interesting to note that both cholestasis and Se toxicity are strongly associated with oxidative damage. The metabolism of Se can lead to redox cycling and produce superoxides, resulting in oxidative stress and lipid peroxidation (Anundi *et al.* 1984; Hoffman *et al.* 1991; Palace *et al.* 2004; Seko *et al.* 1989; Yan and Spallholz 1991). Se can also deplete glutathione, which is crucial in detoxifying the peroxides generated from redox cycling during Se metabolism, as well as other critical cellular functions (Hoffman *et al.* 1989; Hoffman *et al.* 1991; Hoffman *et al.* 1988b; Hoffman 1977). The underlying mechanisms of Se-induced cholestasis are currently unknown and deserve further investigation. Cholestasis is explored in more detail in Appendix A.

Melanomacrophage aggregates (MMAs) in liver of treated fish increased in size and frequency with increasing dietary Se. MMAs have also been observed in livers of juvenile Sacramento splittail (*Pogonichthys macrolepidotus*) exposed to dietary Se (Teh *et al.* 2004). Several other environmental contaminants have also been shown to cause MMAs in the liver and spleen of fish (Akaishi *et al.* 2004; Blazer *et al.* 1987; Vethaak *et al.* 1996; Wolke *et al.* 1985). These aggregates are known to increase in number with age and stress, and are commonly found in mature fish (Agius 1985; Blazer *et al.* 1987; Bonga 1997; Kenedy-Stoskopf 1993; Vethaak and Wester 1996). Consideration of the age of experimental fish is necessary when using MMAs as an indication of physiological stress (Rice 2001; Wester *et al.* 1994). The evaluation of MMAs is appropriate in this

study since juveniles were used and all fish were exposed to identical holding and sampling stressors.

MMAs have a debated function but are generally suspected to be involved in the defense systems of fish (Bonga 1997; Rice 2001; Wester *et al.* 1994). Common pigments in these aggregates (lipofuscin, melanin, hemosiderin, and ceroid) are involved in defensive functions such as hemoglobin degradation, storage of destroyed cellular material, antioxidation, lipid oxidation, and immune function (Rice 2001; Wester *et al.* 1994). The presence of melanin is particularly significant in evaluating Se toxicity considering the importance of this pigment in combating oxidative stress. The finding of increased number and area of macrophage aggregations in juvenile sturgeon fed increasing levels of Se suggests that this element produces active oxygen intermediates and that melanin may be used to combat oxidative damage.

Dietary Se did not appear to cause renal toxicity, based on histopathological analysis. Controls and treated fish showed evidence of generation of new glomeruli and tubules. These results differ from previous studies in other fishes. Hicks *et al.* (1984) found kidney lesions in rainbow trout with liver Se levels similar to those in white sturgeon from this study. Another study found significant tubular changes and signs of glomerulonephritis in kidneys of green sunfish residing in Belews Lake, North Carolina, which is an area of high Se contamination (Sorensen *et al.* 1984). The green sunfish had liver Se levels similar to juvenile sturgeon in the highest treatment of the present study. In addition, striped bass (*Morone saxatilis*) fed Se-laden fish from Belews Lake, and accumulating muscle Se levels similar to those found in our lowest Se treatment, sustained proximal tubule damage of the kidneys (Coughlan and Velte 1989). The lack

of renal toxicity observed in the present study may relate to differences in phylogeny (Chondrostei vs. Teleostei lineages), Se species, exposure route and duration of exposures.

The occurrence of both fused secondary gill lamellae and inflammation of the gills was highest in the low and medium Se dietary treatments (*ca.* 20 & 36 μg/g dietary Se, respectively). These lesions indicate cellular damage (inflammation) and compromised respiration (fused lamellae) in exposed fish. Effects on the gill are a well-documented effect in Se-exposed fish, especially the swelling of secondary lamellae (Lemly 1993a; Sorensen *et al.* 1982a; Sorensen *et al.* 1984). This study provides further evidence that overexposure to Se leads to impaired morphology and function of gill lamellae. However, reasons for the decreased occurrence of gill effects in the highest treatment (*ca.* 53 μg/g dietary Se) are unclear.

There is evidence for several potential mechanisms of Se toxicity in teleosts, birds and mammals. The metabolism of Se can lead to oxidative damage, which can result in damaged tissues (Anundi *et al.* 1984; Seko *et al.* 1989; Yan and Spallholz 1991). Se can also alter natural detoxification systems by depleting glutathione (GSH) or the methylation co-factor S-adenosylmethionine (Anderson and Moxon 1942; Hoffman and Heinz 1988; Hoffman *et al.* 2002; Hoffman 1977). Such alterations can leave the organism more vulnerable to both endogenous and exogenous toxicants. Se can also be incorporated into biological molecules in place of sulfur because the two elements have similar chemical properties (Diplock 1976; Stadtman 1974). However, Se and sulfur bonds may have different strength and function in biological molecules (Reddy and Massaro 1983), thus some Se-substituted compounds may become unstable or

dysfunctional (Lemly 1998; Stadtman 1974). In addition, Se can inhibit protein synthesis (Vernie *et al.* 1974) and react with sulfhydryl groups of proteins or other molecules (Martin 1973). Such cellular disruptions can also lead to tissue damage. Additional research is needed to understand the mechanism(s) of Se-induced toxicity in fishes.

The present study is novel because it exposed the actual species of ecological concern to the Se form (organic Se) and exposure route (dietary) that is predominant in the natural environment. Much of the previous work on Se has utilized waterborne exposures (e.g., Gissel Nielsen and Gissel Nielsen 1978; Hodson *et al.* 1980; Lemly 1982). However, the diet is the most significant route of exposure leading to Se bioaccumulation and toxicity (e.g., Coyle *et al.* 1993; Hermanutz *et al.* 1992; Luoma *et al.* 1992). In addition, the common use of inorganic Se in both waterborne and dietary exposures underestimates environmental exposure, uptake and toxicity of Se to aquatic organisms (Fan *et al.* 2002; Hoffman *et al.* 1989; Wang and Lovell 1997). This work incorporates the species of concern rather than extrapolating potential impacts from experimentation with surrogate species. This is rare in ecotoxicology because it requires the ability to culture a species of ecological concern. For some phylogenetically ancient fish, such as sturgeon, the use of surrogate species is not possible, or is unreliable, because of the significant anatomical, physiological and behavioral differences with modern fish. White sturgeon culture can be difficult and has taken years of research to become widely successful (e.g., Doroshov 1985; Doroshov *et al.* 1997). Were there no aquaculture ponds with the ability to produce sturgeon of spawning maturity, this study would not have been possible.

This work shows that juvenile white sturgeon in San Francisco Bay-Delta are susceptible to toxicity at Se levels currently observed in their prey, and that environmentally feasible increases of Se in this food web leads to increased toxicity in this species. Each of the three dietary treatment levels has environmental relevance for white sturgeon in San Francisco Bay-Delta. The lowest Se experimental diet (*ca.* 20 μg/g Se) is similar to the highest concentrations found in *Potamocorbula amurensis*, which is a common food source of San Francisco Bay-Delta white sturgeon (Linville *et al.* 2002; Luoma and Presser 2000; Stewart *et al.* 2004). The medium and high experimental diets (*ca.* 36 & 53 μg/g Se, respectively) fall within predicted Se concentrations in bivalves resulting from the proposed increase of Se-laden agricultural irrigation discharges into San Francisco Bay-Delta (Luoma and Presser 2000).

Previous studies have shown that juvenile white sturgeon from San Francisco Bay-Delta accumulate high Se levels similar to those found in our experiment. Linares *et al.* (2004) reported a mean liver Se of 9.75 ± 5.35 μg/g in 36 sub-adult and adult (age 4 – 18) white sturgeon sampled in San Francisco Bay between 2002 and 2004 (all measurements of variation reported as standard deviation). Between 1986 and 1990, juvenile white sturgeon captured from this area contained mean Se concentrations of 5.52 ± 3.02 μg/g in muscle and 17.01 ± 15.89 μg/g in liver (n = 18; Urquhart and Regalado 1991), which is similar to the bioaccumulation levels observed in our experiment. It is clear from this data that juvenile white sturgeon in San Francisco Bay-Delta accumulates high, but variable, Se levels that may not induce short-term mortality but could potentially result in the adverse health effects observed in this study.

The direct effects of Se on a white sturgeon population are difficult to detect because monitoring is typically accomplished by tracking sub-adults and adults, which are generally at least 9 years of age (Schaffter and Kohlhorst 1999). Thus, poor recruitment is usually not detected until a decade or more after the hatching of a particular year-class. White sturgeon populations in San Francisco Bay-Delta have been highly variable over the past five decades (Kohlhorst 1980; Kohlhorst *et al.* 1991; Schaffter and Kohlhorst 1999). Schaffter and Kohlhorst (1999) reported a paucity of smaller sturgeon caught in the late 1990's. Population surveys in 2005 indicated that the San Francisco Bay-Delta white sturgeon population was at a 50-year low, with approximately 10,000 adult sturgeon (California Department of Fish and Game Commission 2006). It is important to note that this severe population decrease has occurred approximately a decade after *P. amurensis* significantly increased the availability of Se in San Francisco Bay (Linville *et al.* 2002; Luoma and Presser 2000). If the increased availability of Se in the early 1990's resulted in smaller sturgeon year-classes, we would expect to observe evidence of that impact around this time.

In conclusion, this work shows that juvenile white sturgeon experience significant toxicity upon exposure to elevated dietary Se. In San Francisco Bay-Delta, juveniles are susceptible to toxicity at Se levels currently observed in their prey. Relatively small increases of Se in this food web will lead to increased toxicity to this species. Melanomacrophage aggregates in liver increased in size and number in a dose-response manner between *ca.* 20 to 53 μg/g dietary Se exposures. Cholestasis, a potentially fatal condition, was present in all treatment groups. Exposure to *ca.* 20 and 36 μg/g dietary Se resulted in significant inflammation and fused lamellae in gills, however these effects

occurred less frequently with exposure to *ca.* 53 μg/g dietary Se. Toxicological studies typically utilize a much wider exposure range, with doses differing by orders of magnitude. The narrow exposure range used here provides greater ecological significance; however, the lack of large differences between exposures is not surprising. Field studies have shown that Se is elevated in several branches of the San Francisco Bay-Delta food web, and that white sturgeon in this region have high tissue Se burdens, similar to that in our experiment. The present study indicates that the impacts of Se in this system may be a factor affecting the abundance of white sturgeon in San Francisco Bay. Careful management of all processes with potential to increase food web Se in San Francisco Bay-Delta is essential to sturgeon preservation.

Se content of experimental diets

Diet	Target Se Content (µg/g)	Measured Total Se (µg/g)
Control	1	1.04 ± 0.07
Low	15	20.05 ± 1.10
Medium	30	35.61 ± 3.83
High	45	52.54 ± 2.32

Table 1-1. Se averages ± standard deviation are shown on a dry weight basis (n = 3). Se was added to the diet as selenized yeast (Selenomax®, Ambi Inc.).

Composition of experimental diet

Ingredient:	% of diet
Vitamin Free Casein	31
Wheat gluten	15
Egg White	4
Corn Dextrin	27
Cod Liver Oil	4
Corn Oil	4
Lard	4
Vitamin Premix[1] (w/ cellulose & santoguin)	1
Mineral Premix[2]	3
Cellulose	2.5
Choline Chloride	0.5
Yeast Mix[3]	4

Table 1-2. Experimental diet composition for juvenile white sturgeon. Increasing levels of selenized yeast were used in the treatment diets. Table 1-1 shows the Se level in each diet.

[1]Supplied per 1 Kg diet: 2.69 g Alphacel non-nutritive bulk; 1.47 g Ascorbic acid; 1.47 mg Biotin; 4.91 g Cellulose; 147.2 mg D Calcium pantothenate; 5.3 mg Folic acid 147.2 mg Inositol; 58.88 mg Menadione; 147.2 mg Niacin; 29.44 mg Pyridoxine HCL; 88.32 mg Riboflavin; 186.46 mg Santoguin; 14.72 mg Thiamine Mononitrate; 2.94 mg Vitamin A acetate (500,000 U/g); 58.88 ug Vitamin B-12 crystalline; 98.14 mg Vitamin D3 (400,000 U/g).

[2]Mineral premix was purchase from ICN and the composition was given by Bernhart and Tomarelli (1966).

[3]Yeast mixture contained selenized yeast and Torula yeast.

Se in juvenile white sturgeon after six months of dietary Se exposures
Data displayed as median, 25th - 75th percentiles and full range

Se dietary exposures (µg/g)	Muscle (Se µg/g)			Blood (Se µg/g)			Liver (Se µg/g)		
	Median	25 - 75%	Range	Median	25 - 75%	Range	Median	25 - 75%	Range
1.04 ± 0.07	2.06	1.61 - 2.49	1.36 - 2.76	4.3	3.85 - 5.01	2.14 - 6.90	2.85	2.14 - 4.49	1.31 - 7.35
20.05 ± 1.10	21.56	17.40 - 25.99	13.18 - 32.40	24.04	22.23 - 25.86	18.75 - 33.05	19.49	14.20 - 25.92	7.54 - 53.65
35.61 ± 3.83	39.73	34.55 - 43.58	21.28 - 47.54	40.99	38.19 - 46.25	31.01 - 59.20	40.2	31.48 - 45.32	17.10 - 90.24
52.54 ± 2.32	52.97	46.67 - 59.20	32.06 - 65.59	55.33	51.33 - 63.11	38.52 - 77.90	69.84	60.29 - 98.39	47.02 - 281.64

Table 1-3. Sturgeon data displayed as median, 25th - 75th percentiles, and range of Se (µg/g, dw) levels in liver, blood and muscle after six months of dietary Se exposures. Dietary Se exposure displayed as mean ± standard deviation Se (µg/g, dw). Data displayed in Figure 1-1.

Comparisons of gross morphological characteristics of juvenile white sturgeon following six months of dietary Se exposures

	R^2	F	p-value	Model
Condition Factor	0.15	1.74	0.08	ANOVA
Fork Length	0.09	1.06	0.40	ANOVA
Body Weight	0.10	1.13	0.35	ANOVA
Specific Growth Rate	0.36	1.49	0.29	ANOVA
Gonadosomatic regression	0.59	2.28	0.08	ANCOVA
Hepatosomatic regression	0.58	1.95	0.13	ANCOVA
Weight to length regression	0.84	1.24	0.30	ANCOVA

Table 1-4. Parameters from ANOVA and ANCOVA analyses of gross morphological characteristics between juvenile white sturgeon exposed to *ca.* 1, 20, 36, and 53 µg/g dietary Se for six months. No differences were found for any of the measured characteristics.

Gross morphological measurements of juvenile white sturgeon following six months of dietary Se exposures

Dietary Se (µg/g)	Condition Factor	Fork Length (cm)	Body Weight (kg)	Specific Growth Rate
1.04 ± 0.07	$7.43 \times 10^{-3} \pm 1.7 \times 10^{-4}$ (3)	60.3 ± 0.86 (3)	1.65 ± 0.097 (3)	0.58 ± 0.032 (3)
20.05 ± 1.10	$7.73 \times 10^{-3} \pm 1.7 \times 10^{-4}$ (3)	59 ± 0.39 (3)	1.62 ± 0.051 (3)	0.57 ± 0.02 (3)
35.61 ± 3.83	$7.96 \times 10^{-3} \pm 7.4 \times 10^{-5}$ (3)	59.5 ± 0.32 (3)	1.69 ± 0.039 (3)	0.57 ± 0.016 (3)
52.54 ± 2.32	$7.86 \times 10^{-3} \pm 2.8 \times 10^{-4}$ (3)	56.4 ± 0.66 (3)	1.45 ± 0.103 (3)	0.51 ± 0.035 (3)

Table 1-5. Measurements of weight, length, condition and growth in juvenile white sturgeon for each dietary exposure level. All data is presented as mean ± standard error (number of tank replicates). No statistical differences were found between dietary exposure levels (Table 1-4).

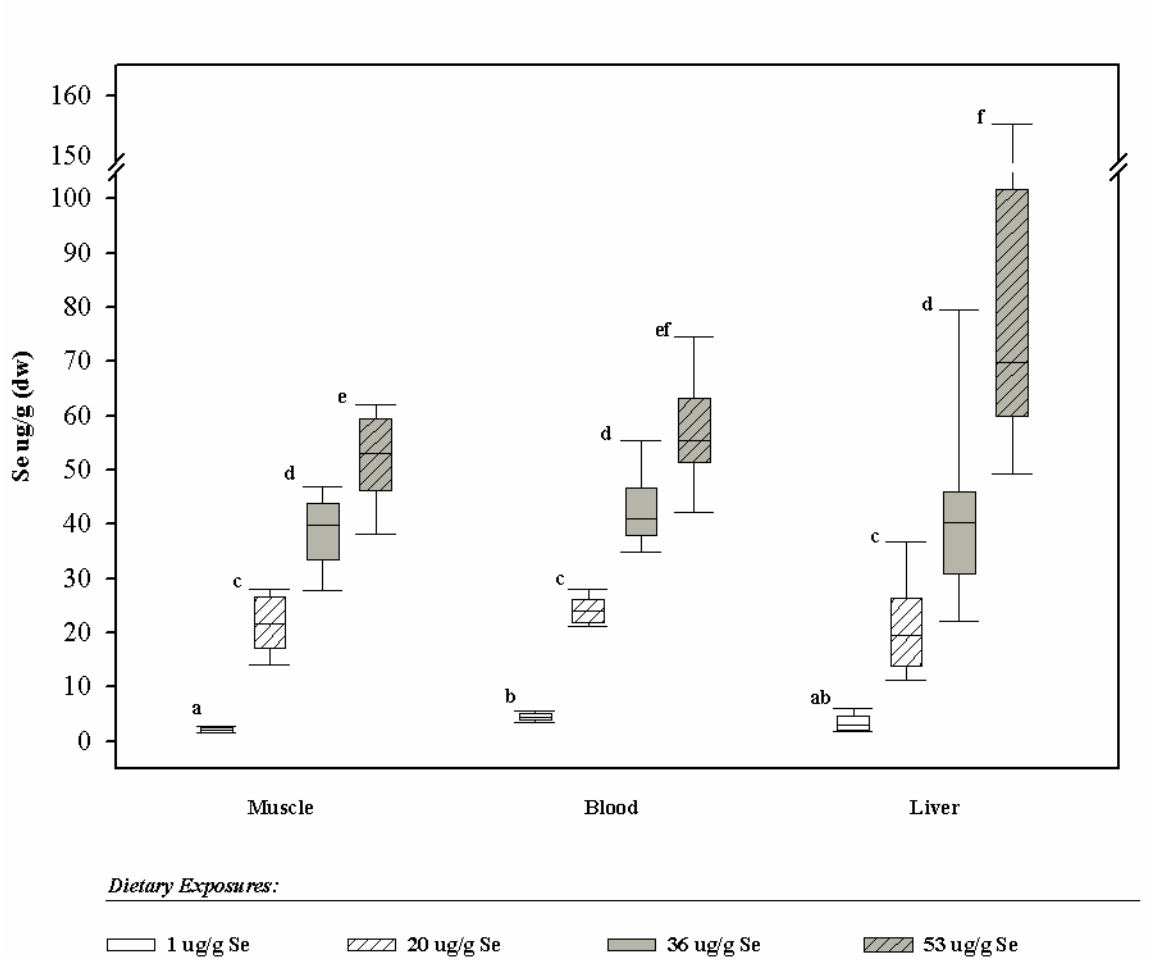


Figure 1-1. Se levels in liver, blood and muscle after six months of dietary Se exposures. For each tissue type, Se increased with increasing dietary Se exposure (non-parametric Rank F-Test, $p = 0.03$, adjusted multiple comparisons, $\alpha = 0.05$). In general, muscle accumulated less Se than either liver or blood. Standard box and whiskers plots are used to describe these non-parametric data. The horizontal mid-line represents the median while the upper and lower horizontal lines represent the 25th and 75th percentiles, respectively. The whiskers show the 10th and 90th percentiles. Values labeled with the same letters are not statistically different (non-parametric Rank F-Test, $p = 0.03$; adjusted multiple comparisons, $\alpha = 0.05$).

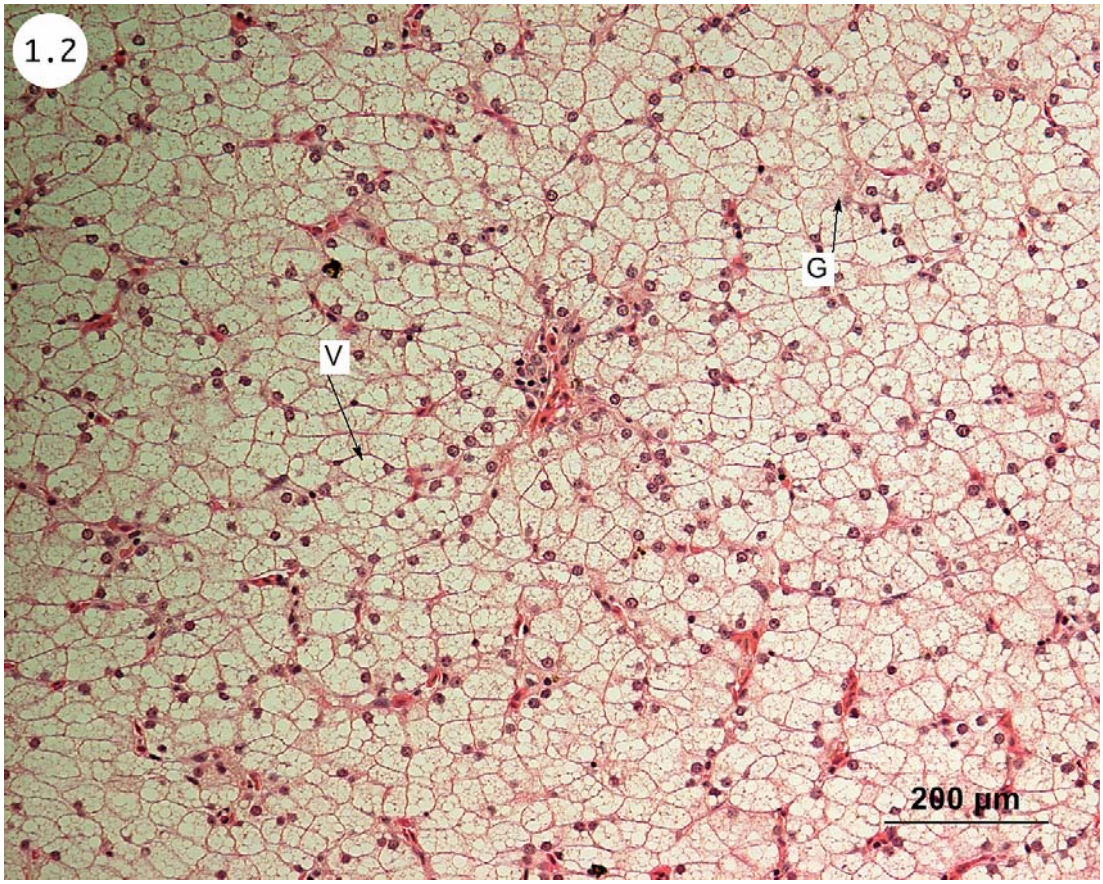


Figure 1-2. Section of liver from control group of juvenile sturgeon. Portal venule is at center of field and is associated with a small bile duct (to left of venule). Hepatocyte polarity is shown by localization of nucleus near basal (sinusoidal) plasma membrane in base of cell (horizontal arrows) and apex directed toward center of hepatic tubule (vertical arrows). Vacuolar profiles (V) in hepatocytes represent sites of lipid while glycogen stores (G) stain more intensely and show no vacuolation. Some hepatocyte profiles do not show a nucleus due to the large difference between cytoplasmic and nuclear volumes.

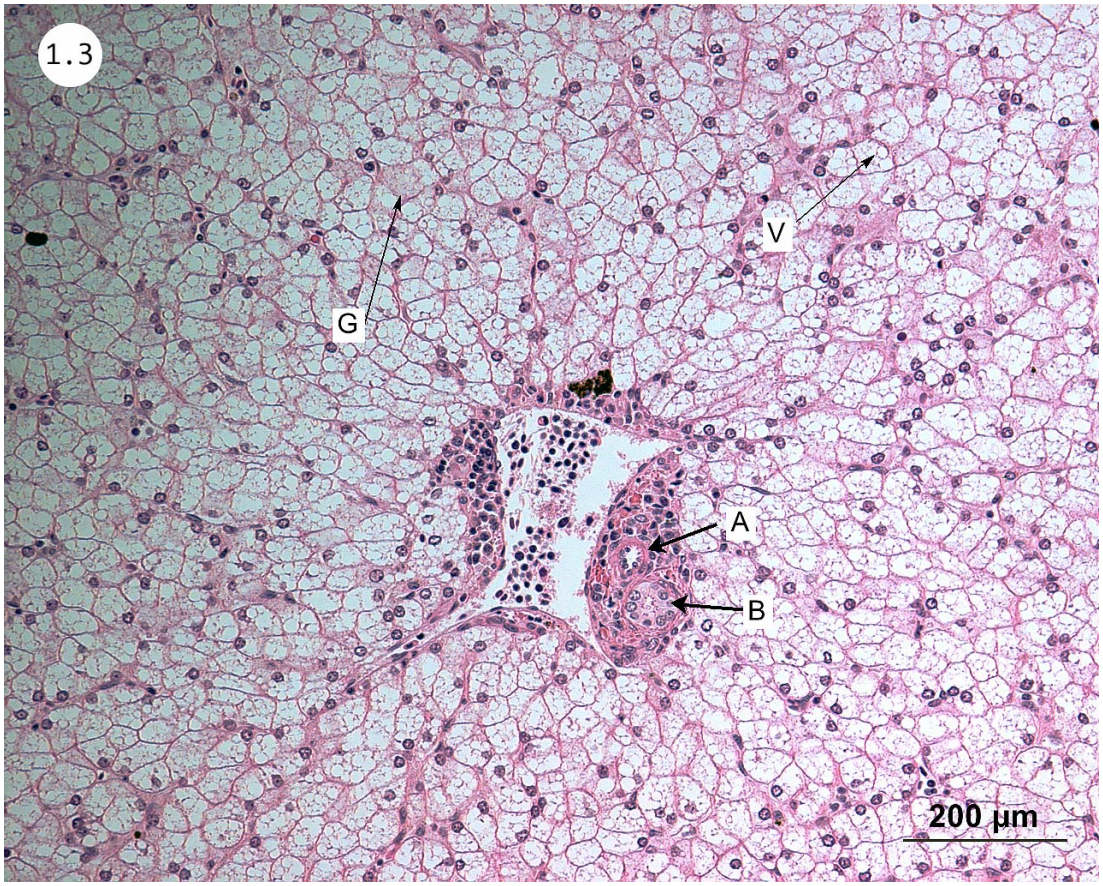


Figure 1-3. Additional micrograph from section of control liver shown in Figure 1-2. Conducting portal vein is shown in center of field and is closely associated with small bile duct (B) and hepatic arteriole (A). Mononuclear leukocytes form cuff around vein. Glycogen storage areas (G) and areas of lipid vacuolation (V) are shown in hepatocytes.

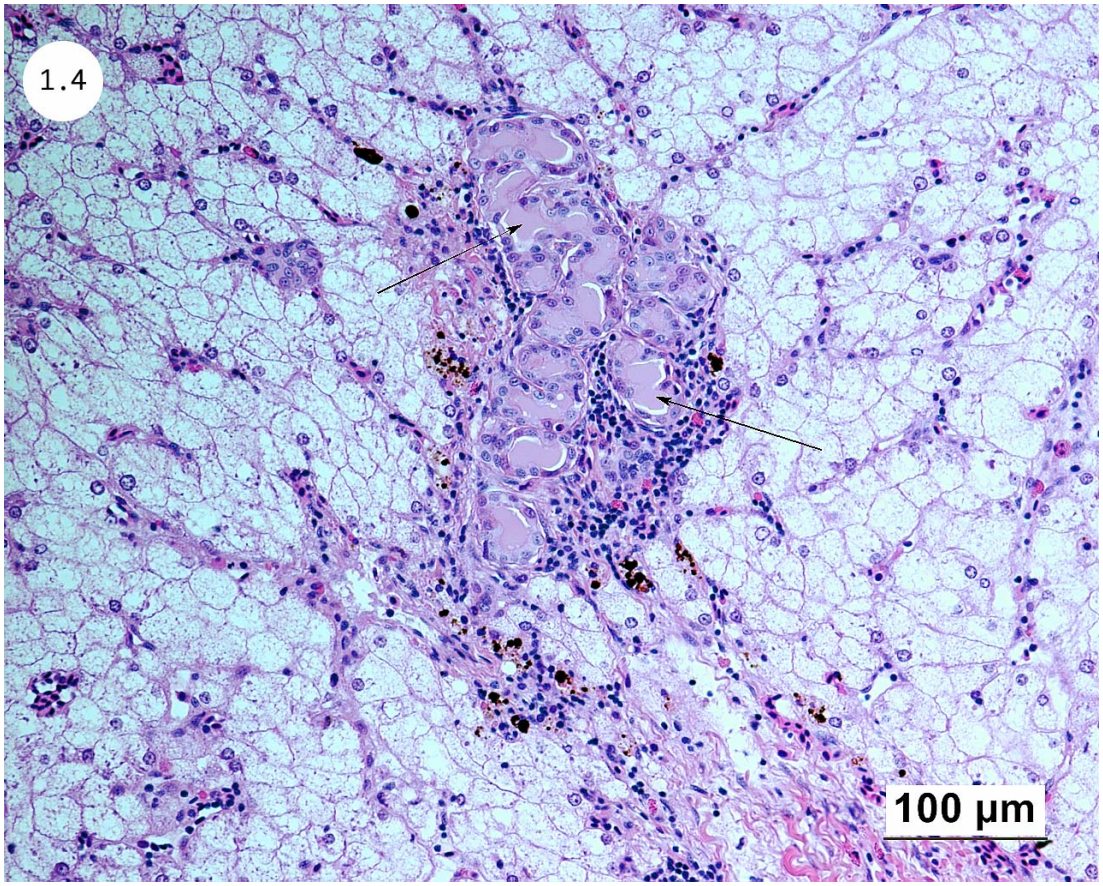


Figure 1-4. Section of liver from control group of juvenile sturgeon showing bile retention (arrows) and focus of small bile duct proliferation. This condition was seen in a single individual. Note absence of evidence for biliary stasis involving canalicular network.

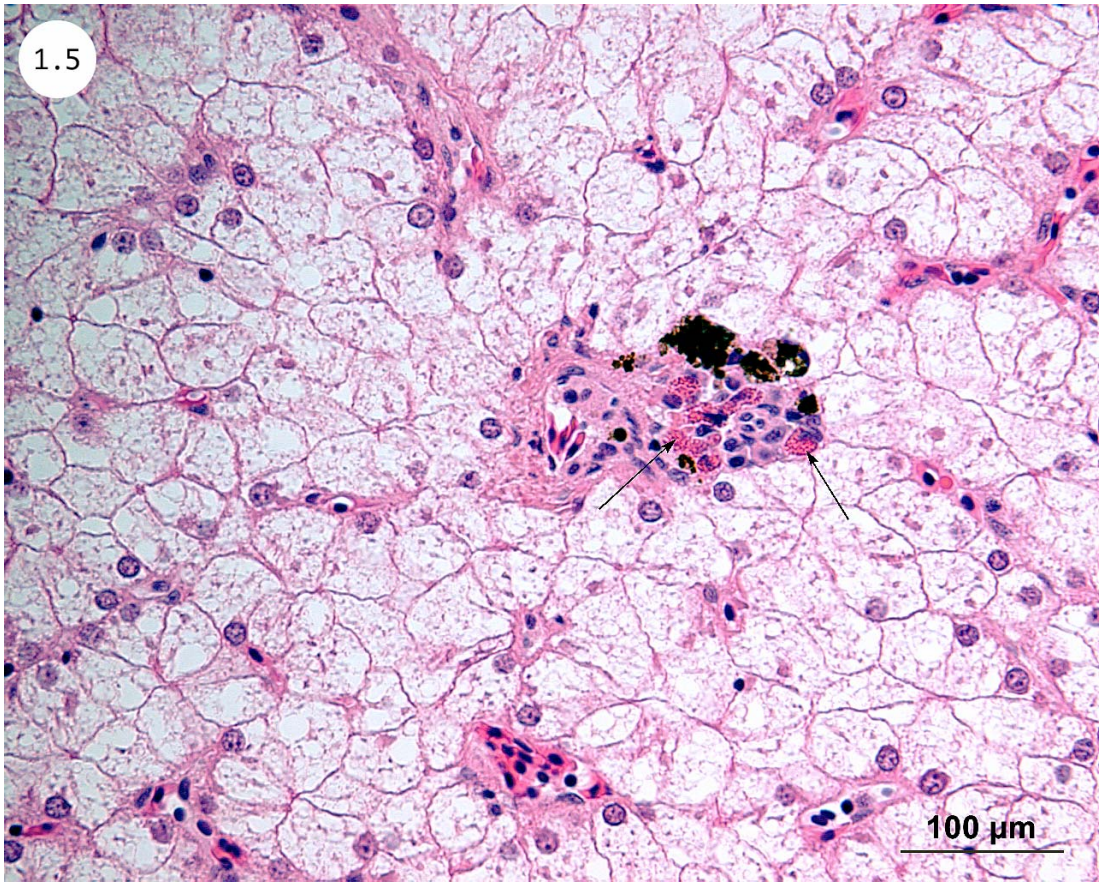


Figure 1-5. Section of liver from control group of juvenile sturgeon. Eosinophilic granular leukocytes (arrows) are associated with portal venule and small bile duct.

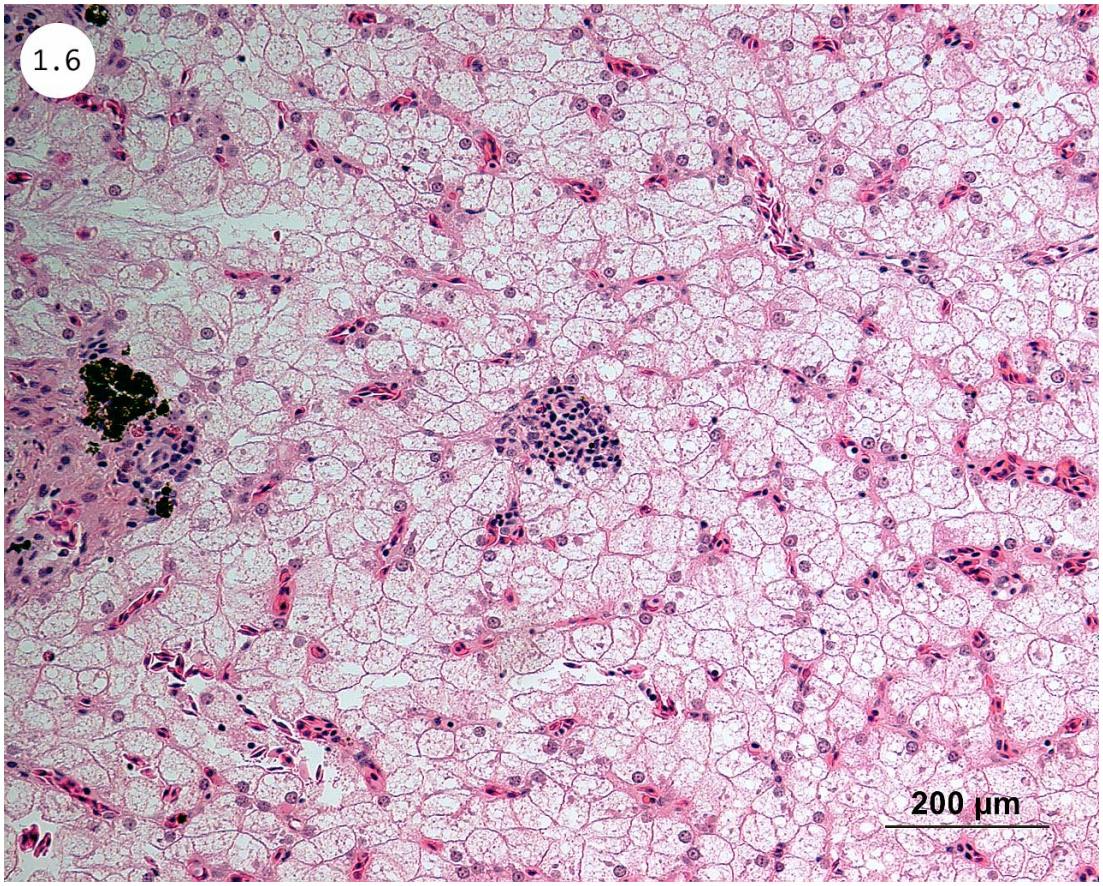


Figure 1-6. Section of liver from control group of juvenile sturgeon. Comparison of this micrograph with figures 1-2 and 1-3 illustrate range of features in livers of control group. A single focus of inflammation involving mononuclear leukocytes is shown in center of field. To left of field, melanomacrophage aggregate is larger than those of Figure 1-3 and is associated with inflammation around a small bile duct.

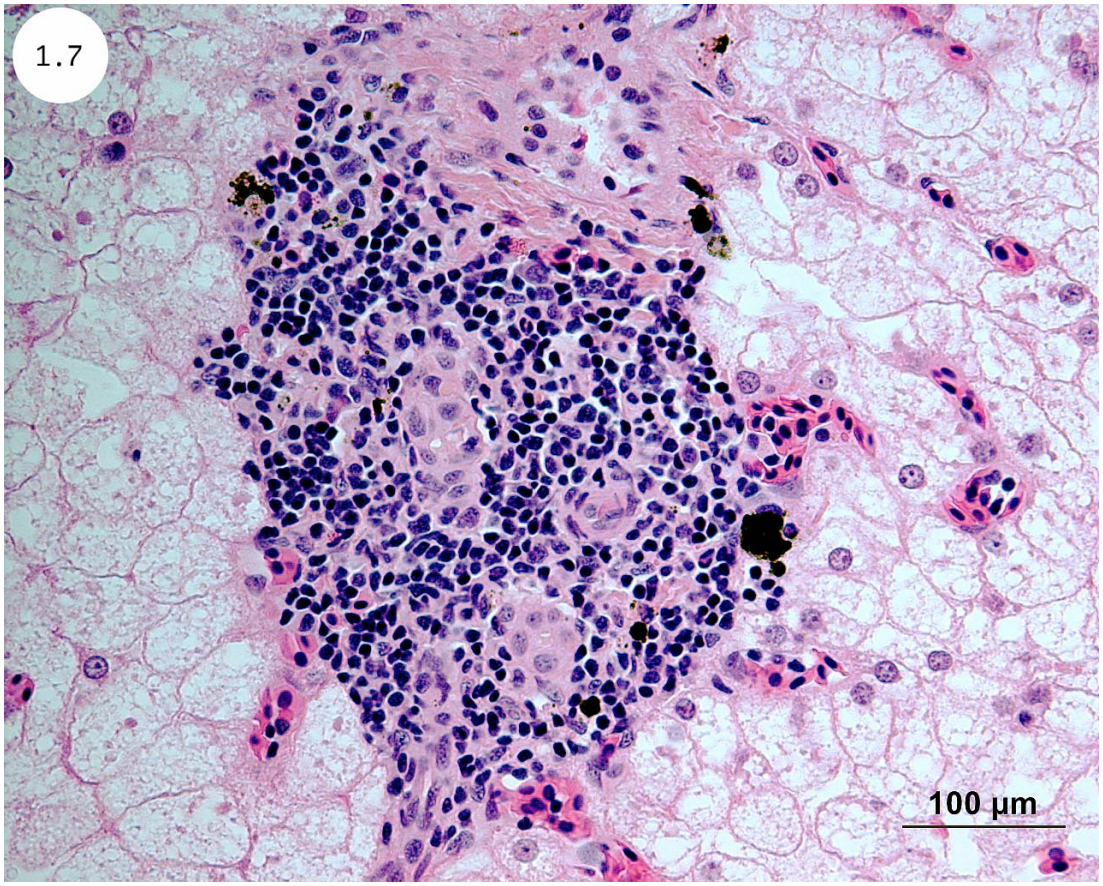


Figure 1-7. Section of liver from control group of juvenile sturgeon showing most extreme case of peribiliary inflammation (mononuclear leukocytes). This type infiltration was consistently seen near small bile ducts and often where these were closely associated with arterioles.

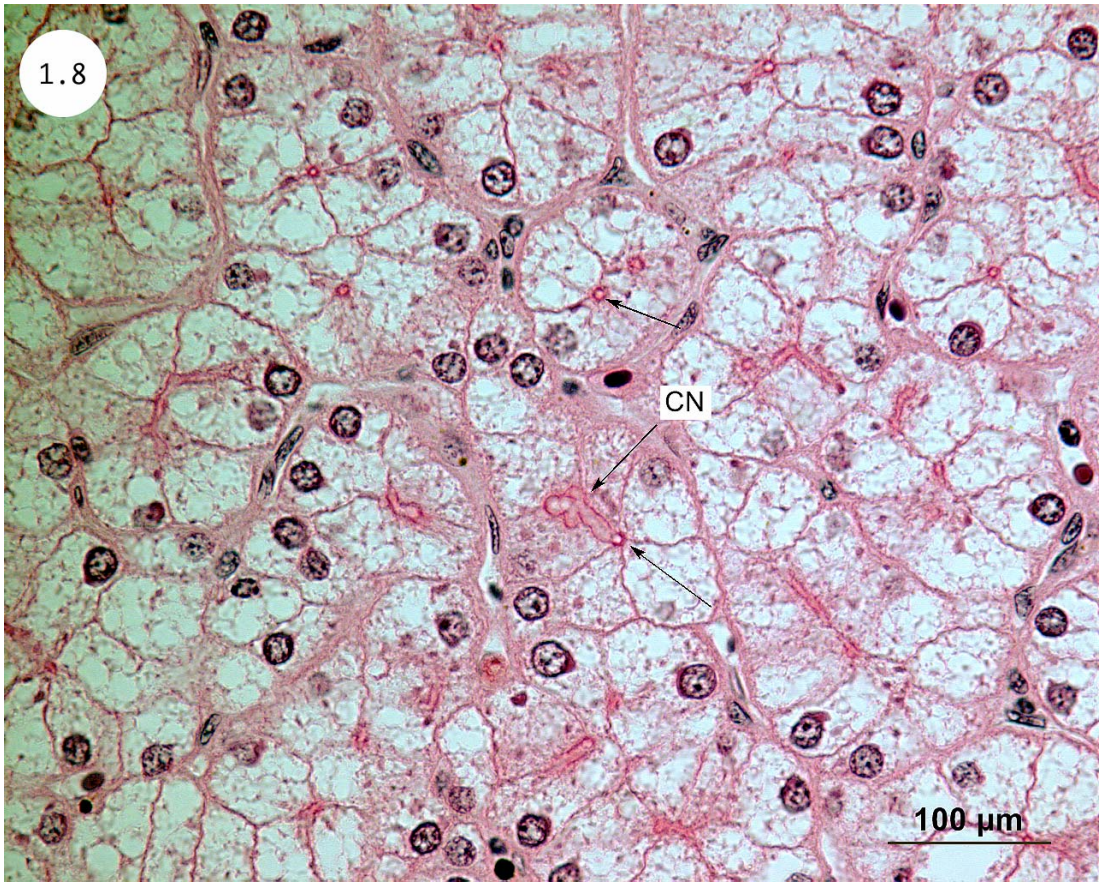


Figure 1-8. This individual was fed 20.05 ± 1.10 µg/g Se in diet for 6 months. Red circle in center of transversely sectioned hepatic tubule is a canaliculus (small arrow). Longitudinal arrays of canalicular network are shown (CN). These features were not visible in juveniles of control group (Figures 1-2 – 1-7).

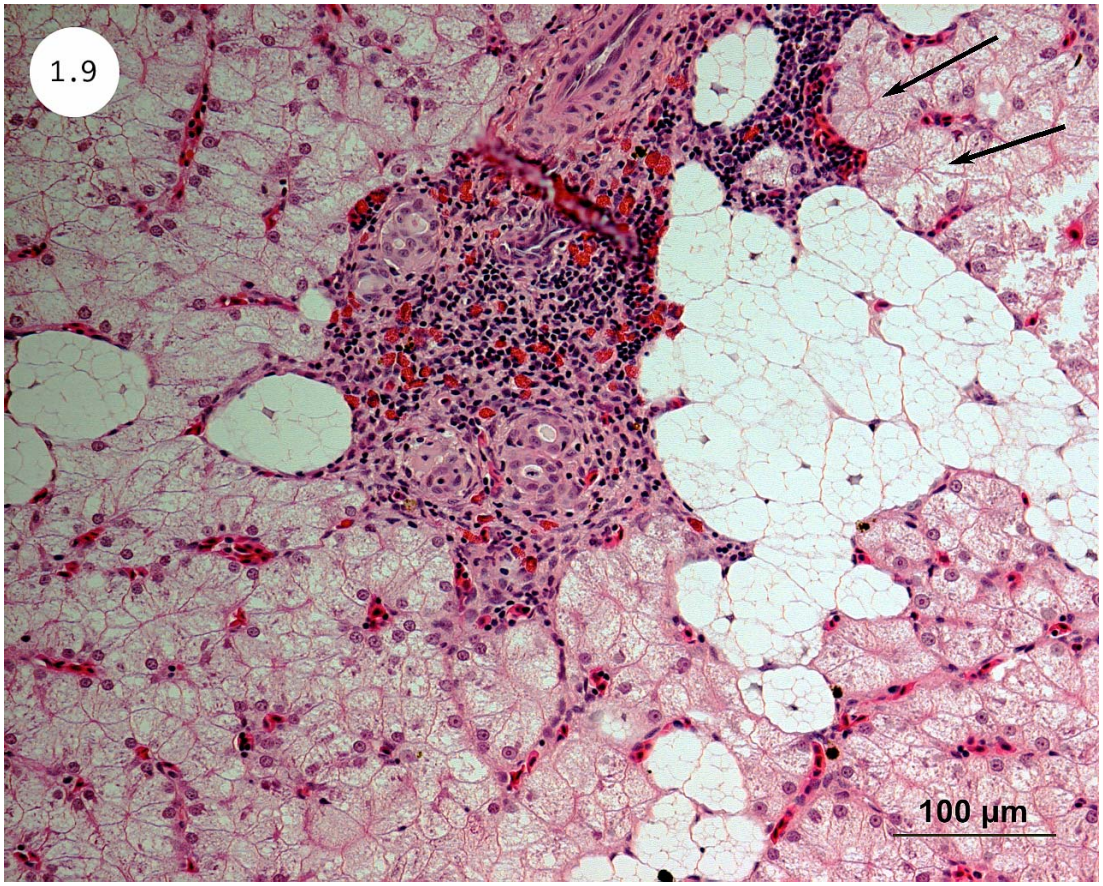


Figure 1-9. This individual was fed 20.05 ± 1.10 µg/g Se in diet for 6 months. High amplitude swelling of hepatocytes (large white cellular arrays) in tubule give rise to tubuloform swelling that was seen at 30% incidence in this group. This condition was seen in only one of 30 control juvenile sturgeon. Two normal appearing hepatic tubules in transverse section are shown (arrows). Inflammatory cells at center of field surround 8 profiles of small bile ducts (bile duct proliferation).

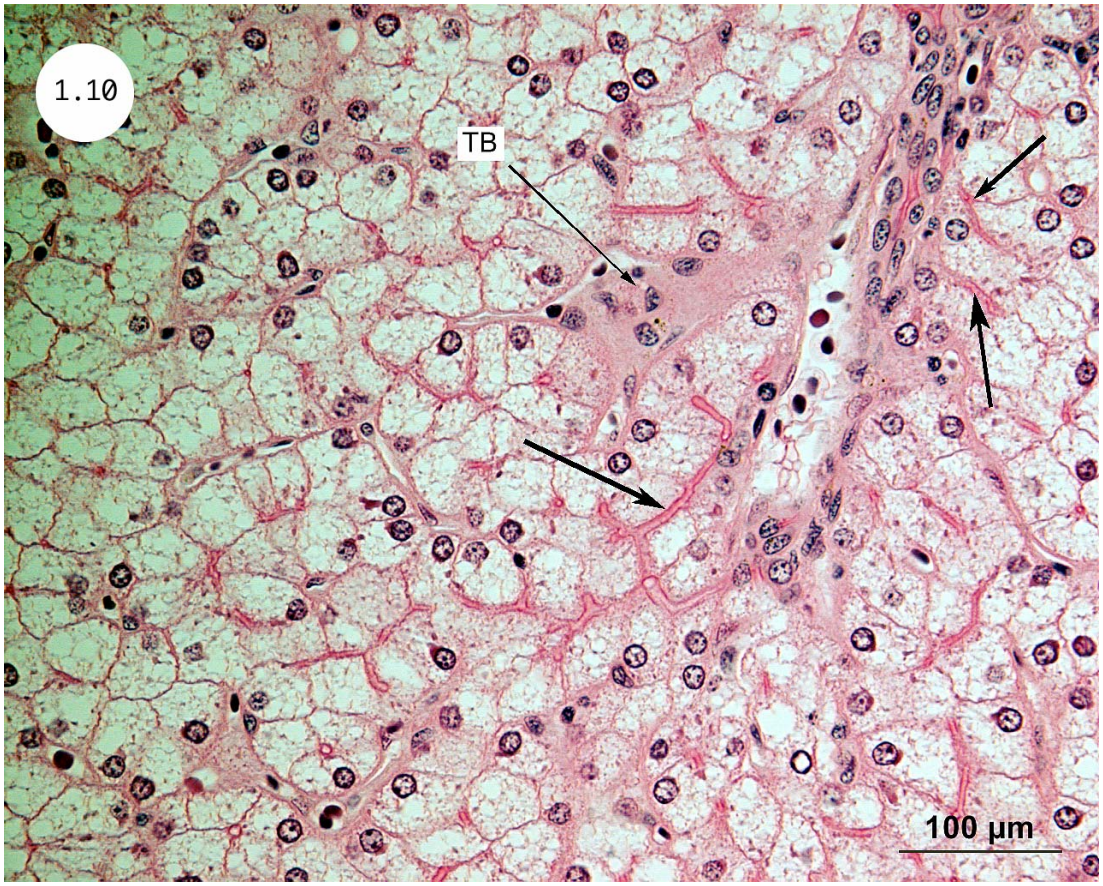


Figure 1-10. This is an additional view of the individual in Figure 1-8. Continuation of canaliculi into areas of transitional biliary epithelial cells and cholangioles is shown (large arrows). Longitudinal array of bile ductule shows apical margins of cells with staining identical to that of canaliculi. In addition, a site of transitional biliary epithelial cell proliferation is seen (TB).

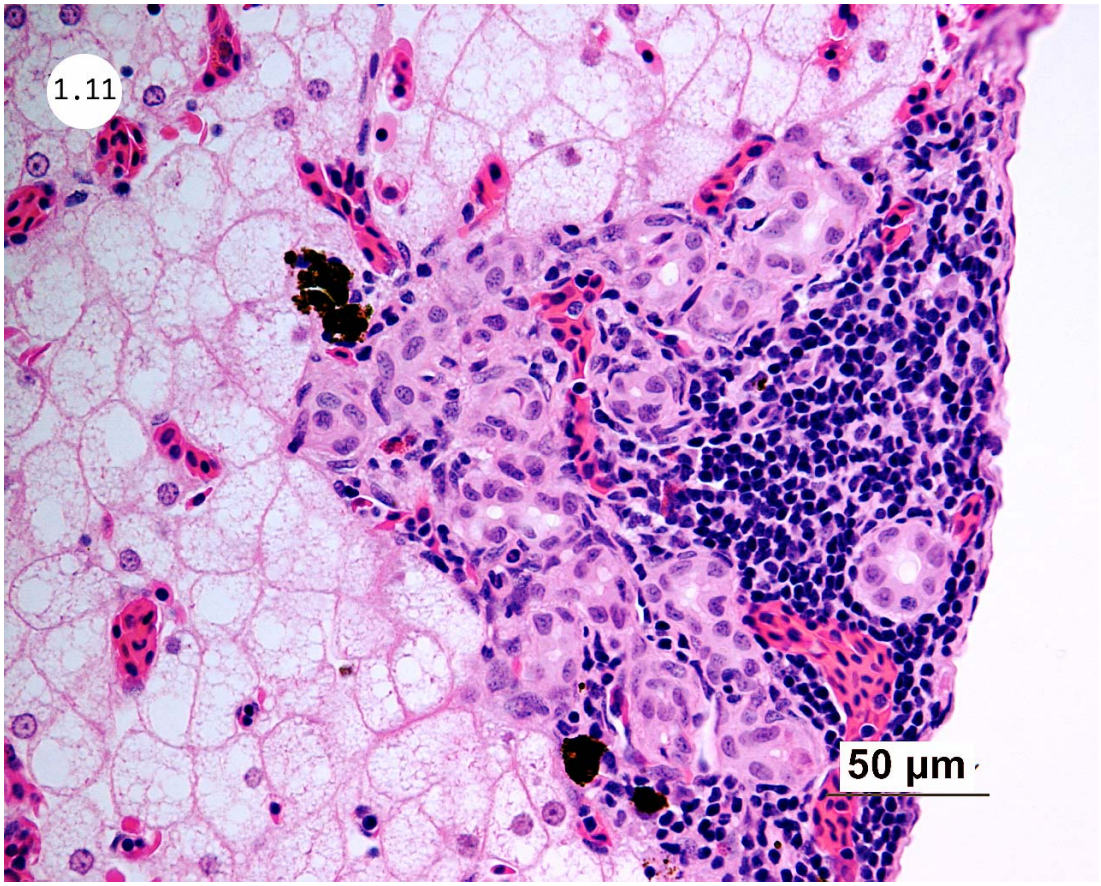


Figure 1-11. This individual was fed 20.05 ± 1.10 µg/g Se in diet for 6 months. Focus of bile duct proliferation is shown with inflammatory cells just beneath hepatic capsule.

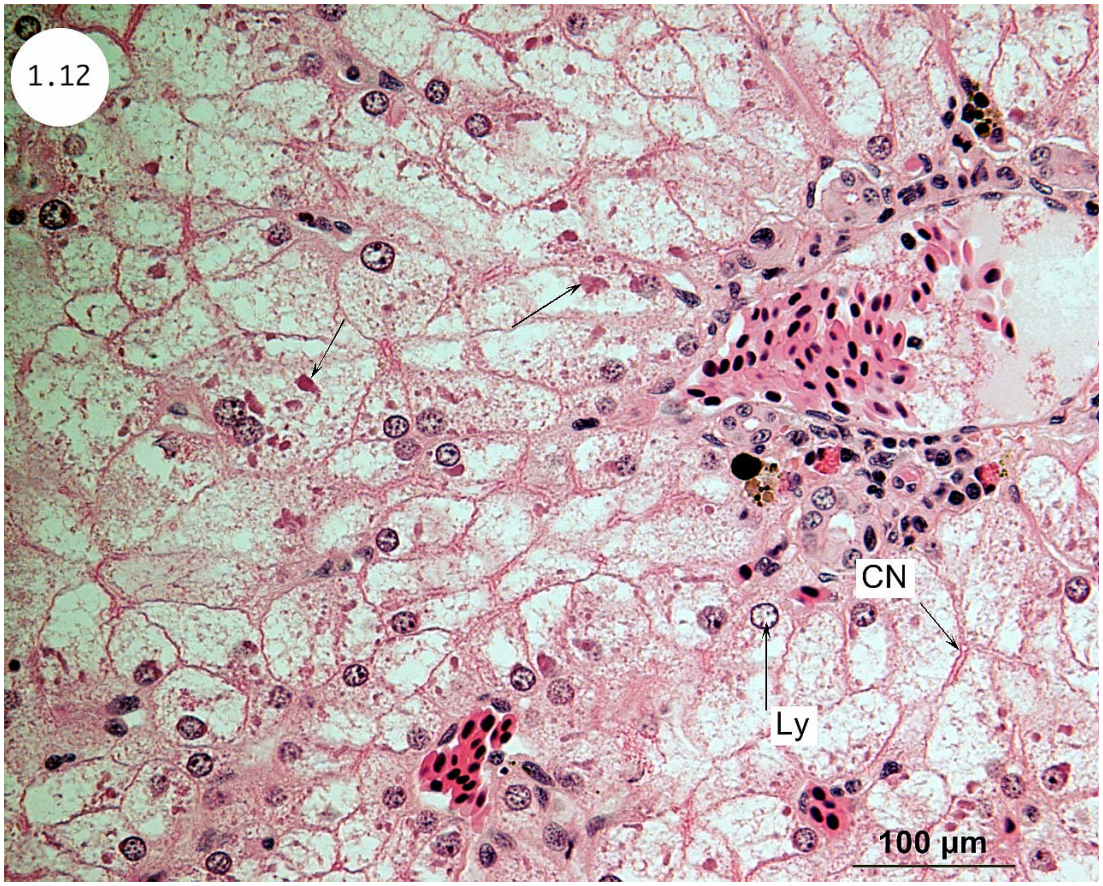


Figure 1-12. This individual was fed 35.61 ± 3.83 µg/g Se in diet for 6 months. This micrograph shows morphology of portal venule and adjacent hepatic parenchyma. Hepatocytes show signs of necrosis seen as nuclear lysis (Ly). In addition, cells are swollen and contain discreet particulate material in cytoplasm (arrows). Canalicular network is distended (CN). In addition there are four profiles of transected small bile ducts around the venule.

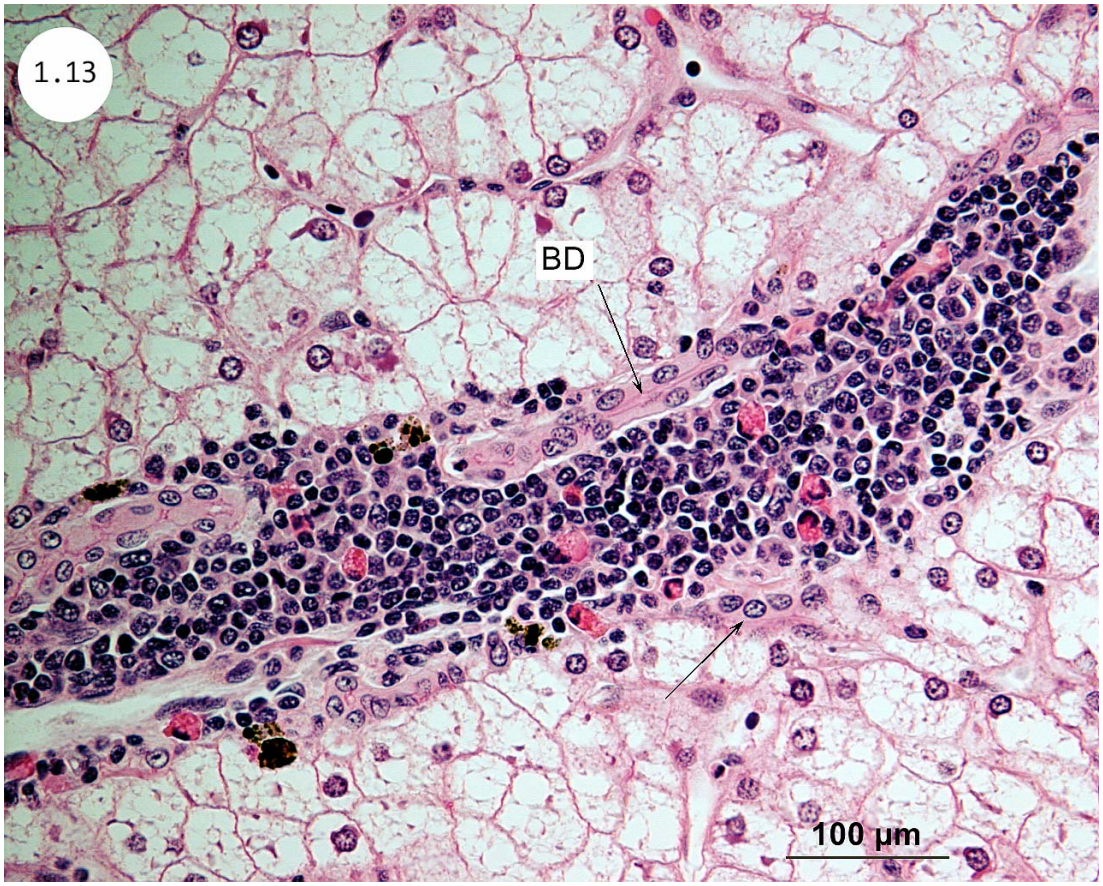


Figure 1-13. This individual was fed 35.61 ± 3.83 µg/g Se in diet for 6 months. Extensive perivenous (venular lumen is at right of field) and peribiliary inflammation involving predominantly mononuclear leukocytes with occasional eosinophilic granular leukocytes. Hepatocytes appear swollen and a single, bright-red Councilman body is seen in perisinusoidal macrophage at top of field. Biliary epithelial cell proliferation is seen (arrow) with cells extending from perivascular site into adjacent parenchyma. Also, note the longitudinal arrays of biliary ductules with red staining in apical plasma membrane region.

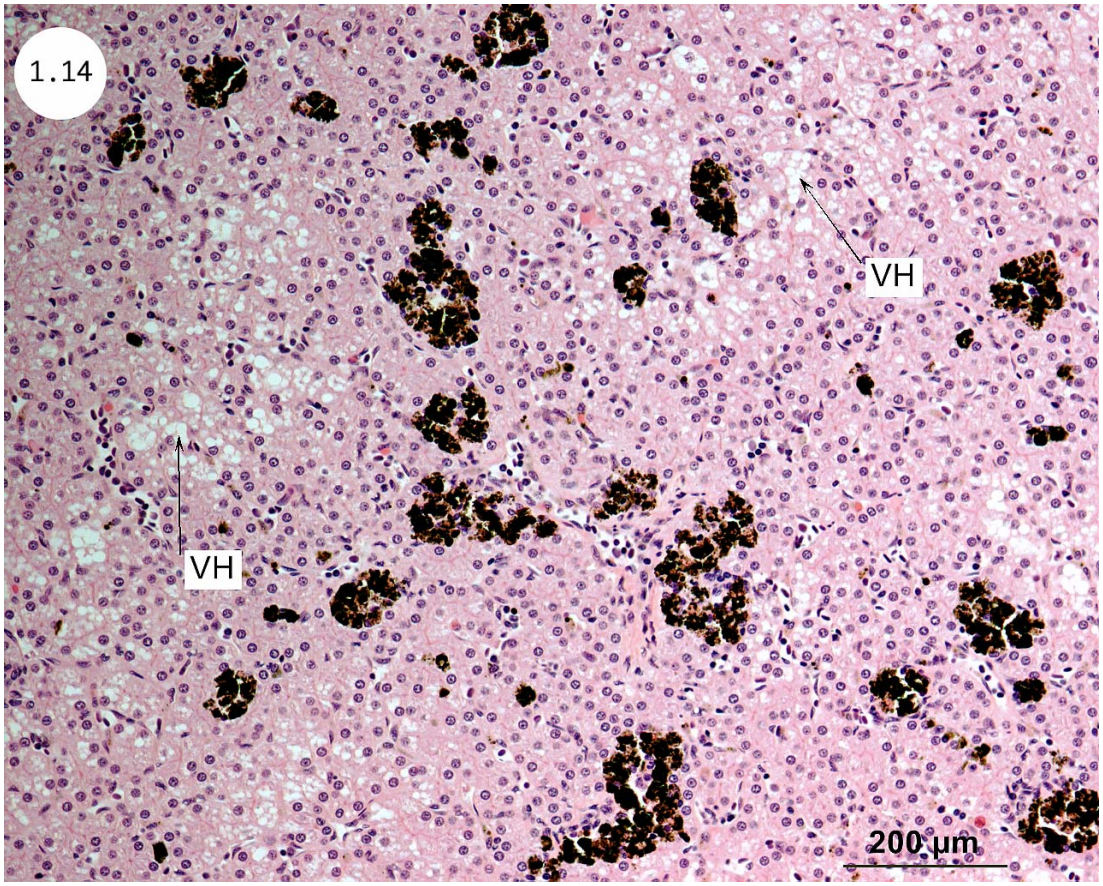


Figure 1-14. This individual was fed 52.54 ± 2.32 µg/g Se in diet for 6 months. This field is from liver of individual showing maximum response of melanomacrophage aggregates. The number of hepatocyte nuclei per area of parenchyma is greatly enhanced and the cytoplasm stains with more intensity. This indicates that hepatocytes have become smaller in volume. Under this level of magnification, a mosaic pattern is evident. Vacuolated hepatocytes occur in zones that are free of melanomacrophage aggregates while hepatocytes, adjacent to macrophage aggregates, are free of vacuolation, smaller (nuclei more abundant), and stain more intensely.

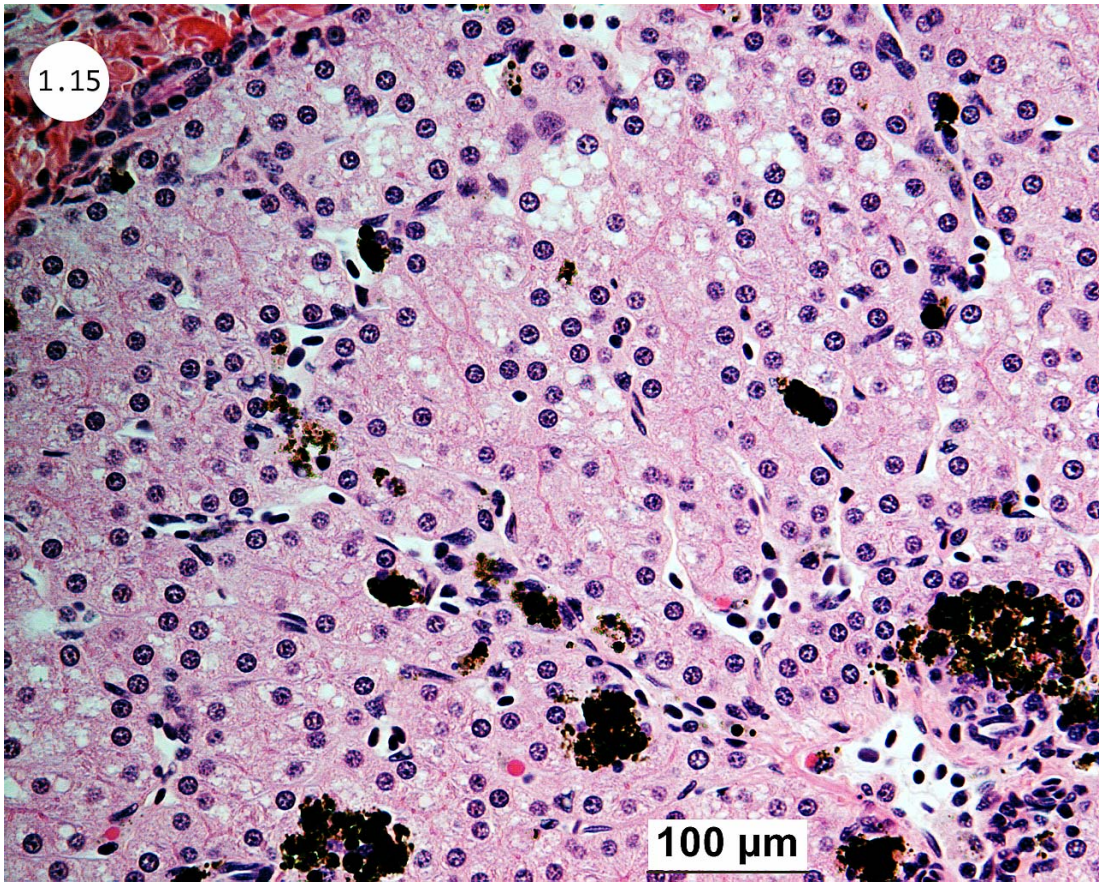


Figure 1-15. This higher magnification view of area near that of Figure 1-14 shows features in greater detail. Note vacuolation of hepatocytes in upper half of field. This is consistent with lipid change. Non-vacuolated hepatocytes (lower portion of field) are smaller; and, profiles of hepatic tubules (T) are more obvious. Hepatic tubules reveal canalicular network (see red lines in center of tubules). Melanomacrophage aggregates of various sizes are prominent. Councilman bodies are seen in four cells in bottom half of field.

Effects observed in gill of juvenile white sturgeon following six months of dietary Se exposure

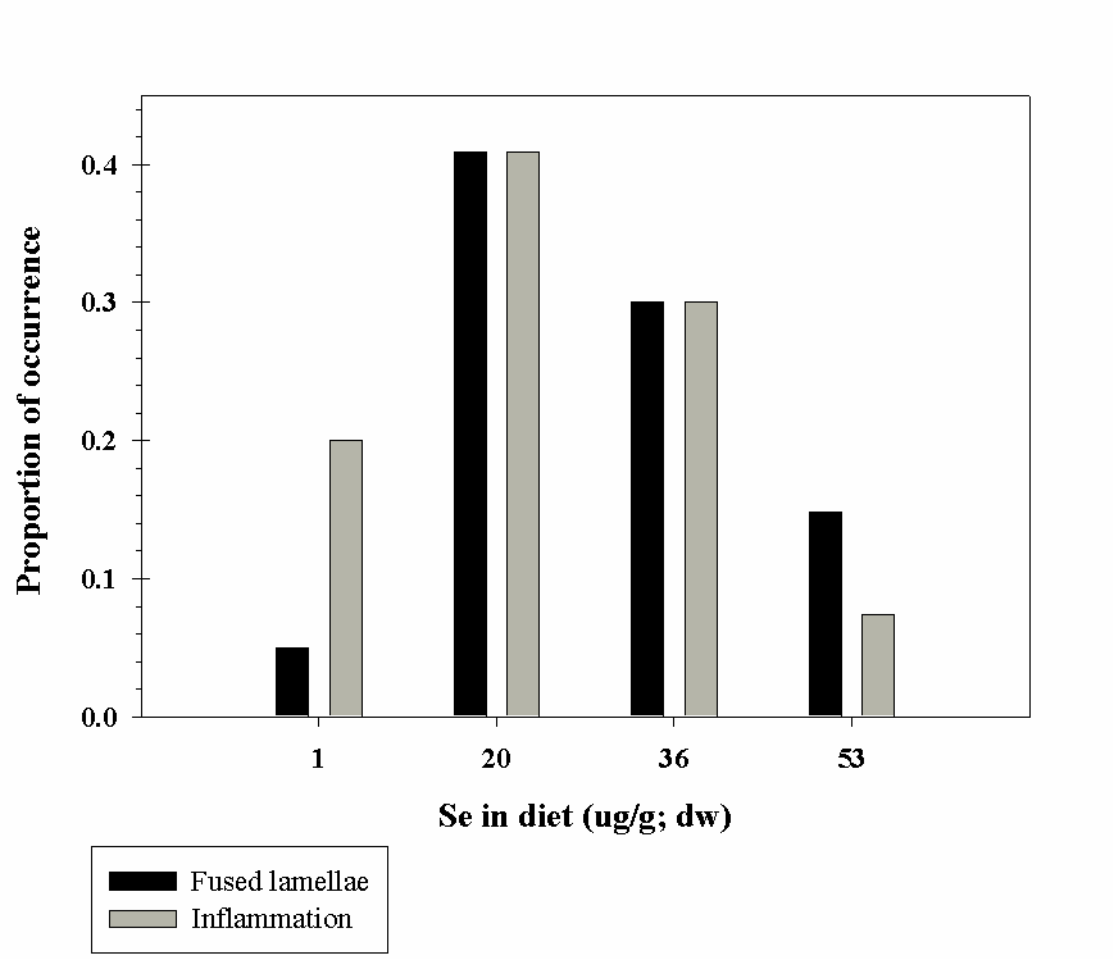


Figure 1-16. Contingency table showing the occurrences of inflammation and fused lamellae in gills of juvenile white sturgeon exposed to dietary Se for six months. The occurrence of inflammation and fused gill is significantly different across treatment levels ($p = 0.041$ and 0.024, respectively). The low and medium treatments contribute the most to this difference.

Chapter 2. Dose-dependent maternal transport of selenium via vitellogenin in white sturgeon

Introduction

Selenium (Se) is a well-documented developmental toxicant to fish and wildlife species (Coyle *et al.* 1993; Gillespie and Baumann 1986; Hamilton 2004; Hamilton *et al.* 1986; Hermanutz 1992; Hoffman *et al.* 1988b; Lemly 1993b; Ohlendorf *et al.* 1986). In small quantities (0.1 – 1.25 μg/g diet) Se is an essential nutrient for animals, humans and many micro-organisms (Diplock *et al.* 1976; Gatlin and Wilson 1984b; Hilton *et al.* 1980; Mayland 1994). However in slightly greater quantities (as little as 13 μg/g Se in diet) this element can impair fish reproduction and development (Woock *et al.* 1987). Excess Se has led to severe teratogenesis, mortality and endangerment or extirpation of fish species residing in areas receiving wastewaters from the combustion of fossil fuels (Garrett and Inmann 1984; Lemly 1985) or drainage from seleniferous land (Hamilton 1999; Skorupa 1998).

Higher trophic level species are most threatened by Se in aquatic ecosystems due to the efficient food web transfer and biomagnification of this element (Coughlan and Velte 1989; Lemly 1985; Luoma *et al.* 1992). The predominant pathway of Se transfer in the aquatic food web is through the assimilation of dissolved Se by phytoplankton, which biotransform inorganic Se into organic Se (Hu *et al.* 1997; Luoma *et al.* 1992; Vandermeulen and Foda 1988; Wrench 1978). Organic Se is accumulated from phytoplankton by filter feeding bivalves (Luoma *et al.* 1992) and is consequently transferred to secondary consumers, such as fish foraging on bivalves. Several studies

have shown the efficient bioaccumulation of Se in fish exposed to a range of concentrations (Cleveland *et al.* 1993; Crane *et al.* 1992; Hamilton *et al.* 1990b; Hamilton *et al.* 2002b; Hilton *et al.* 1980).

Bioaccumulated Se in maturing fish can be passed to the egg yolk, causing teratogenesis of developing embryos and complete reproductive failure (Coyle *et al.* 1993; Gillespie and Baumann 1986; Lemly 1993b; Schultz and Hermanutz 1990). The maternal transport of contaminants to offspring is common in aquatic species. The yolk protein precursor vitellogenin (VTG), synthesized in the liver, has been shown to be the vehicle of maternal transport for several pollutants (Falchuk and Montorzi 2001; Ghosh and Thomas 1995; Monteverdi and Di Giulio 2000). A previous study on white sturgeon has indicated that VTG may also transport Se to the egg yolk (Kroll and Doroshov 1991). Se has chemical properties similar to sulfur and can substitute for sulfur in biological molecules (Diplock 1976; Stadtman 1974). In the case of VTG, Se may be substituted in the sulfur amino acids, methionine, cysteine, and cystine present in the backbone of the VTG molecule (Bidwell and Carlson 1995; Kroll and Doroshov 1991).

Estrogen induces synthesis of VTG in liver, after which VTG is transported by the circulatory system to the growing oocytes (eggs) where it is selectively taken in by receptor-mediated endocytosis (Mommsen and Walsh 1988). In the egg cytoplasm the VTG is broken up and accumulated as egg yolk proteins, phosvitin and lipovitellin, which are stored as nutrients for the embryo. Kroll and Doroshov (1991) measured Se levels in plasma (5 – 9 μg/g), VTG (3 – 4 μg/g), ovulated eggs (3 – 29 μg/g), phosvitin (3 – 90 μg/g) and lipovitellin (3 – 11 μg/g) from six wild-caught white sturgeon females from San Francisco Bay-Delta (dry weight). These elevated, although variable, levels

suggest that VTG is a potential vehicle for the transport of Se to the egg yolk of this valuable endemic species of sturgeon.

White sturgeon are especially vulnerable to Se bioaccumulation and maternal transport due to benthic feeding habits, a long life span, and a prolonged (two year) period of vitellogenesis and yolk deposition (Doroshov 1985; Doroshov *et al.* 1997). White sturgeon is common in San Francisco Bay-Delta, an area with known Se contamination. San Francisco Bay-Delta receives Se through both the refining of fossil fuels and irrigation drainage from the seleniferous San Joaquin Valley. White sturgeon in this area are exposed to high levels of Se through their diet. This is evidenced by high tissue Se levels in common prey of white sturgeon (Linville *et al.* 2002; Stewart *et al.* 2004; White *et al.* 1988), as well as in sturgeon muscle, liver and eggs (Kroll and Doroshov 1991; Stewart *et al.* 2004; Urquhart and Regalado 1991; White *et al.* 1989).

The objectives of this experimental study were to verify the mechanism of maternal transport of Se in sturgeon, and to evaluate the bioaccumulation, maternal transport, and reproductive success in white sturgeon exposed to elevated dietary Se. Two groups of eight cultured white sturgeon females in the advanced stage of vitellogenesis were fed diets with normal (*ca.* 1.42 μg/g) or elevated (*ca.* 34.04 μg/g) Se (as selenized yeast) for approximately 6 months. Selenized yeast, predominately in the form of selenomethionine (Polatajko *et al.* 2005), was used as a surrogate for organic Se present in the aquatic environment. Organic forms of Se, particularly selenomethionine, have been shown to have greater bioavailability and toxicity than inorganic forms and are often the species to which aquatic organisms are exposed (Hamilton *et al.* 1990b; Hoffman *et al.* 1989; Luoma *et al.* 1992; Reinfelder *et al.* 1997; Wang and Lovell 1997).

Upon acquisition of maturation competence, the fish were induced to spawn and ovulated eggs were fertilized with sperm from non-treated males. Fecundity, fertilization, and neurulation rates were measured as indicators of reproductive success. Development of embryos and yolk sac larvae was monitored as part of a separate experiment described in Chapter 3 of this dissertation. Adult females were necropsied for Se analysis. We hypothesized that VTG is the vehicle of maternal transport in fish, as indicated by Kroll and Doroshov (1991; Figure 2-1). We also hypothesized that exposure to elevated dietary Se leads to significant bioaccumulation, maternal transport and reproductive impairment in white sturgeon.

Materials and Methods

Source of Animals

Sixteen virgin females in mid-vitellogenic stage of oogenesis were obtained from The Fishery, Inc. (Galt, California) in September 2001. These sturgeon were from a 1995 year-class and had been reared from birth in circular tanks (with increasing diameter to accommodate growth) with flow-through well water (20 – 21 ºC). During the previous year and a half, they had been cultured in 12.2 m diameter concrete tanks, at densities of 60 to 80 kg/m^3, and fed a commercial high-energy salmonid diet (45% protein/25% lipid) at approximately 0.25% body weight/day.

The sturgeon were transported to the Putah Creek Hatchery Facility (PCHF) at University of California, Davis (UCD) on September 26, 2001 (1-hour travel time). Upon arrival, each fish was anesthetized in 50 mg/L MS-222 until a stage 4 to 5 anesthesia was achieved. A small incision was made in the abdomen to allow insertion of

a catheter for the collection of eggs, which were placed into Ringers solution for measurement of egg diameter and weight. Blood was collected from the caudal vasculature, using a 10 ml heparinized Vacutainer®, and weight and fork length were measured. Each female was PIT tagged in the caudal peduncle region under a lateral scute and externally marked with a floy tag. The fish were randomly assigned to two 6.1 m diameter tanks, with eight in each tank.

Experimental Design

Domestic broodstock white sturgeon females (mean weight and fork length: 22.71 Kg and 134.59 cm) were held in two 6.1 m diameter outdoor flow-through tanks at PCHF. The experiment consisted of two dietary treatments: control (1.42 ± 0.03 μg/g Se) and treatment (34.04 ± 0.82 μg/g Se; Table 2-1). Each tank held 8 females and received one of the two experimental diets for approximately 6 months. Upon acquisition of maturation competence, the fish were induced to spawn and ovulated eggs were fertilized with sperm from non-treated males. Fecundity, fertilization and neurulation were measured as indicators of reproductive success. Females were necropsied for Se analysis following spawning or the failure to ovulate.

Initiation and maintenance

The sturgeon were maintained on well water (18-19 ºC) for approximately 2 months following their arrival at UCD and were slowly transitioned to 100% surface irrigation water in December, 2001, in order to prevent ovarian follicular atresia (Linares-Casenave *et al.* 2002). The irrigation water source typically cools down to 11 to 12 ºC in

Dec to Jan and gradually warms to 15 to 16 ºC in April-May. Water flows were approximately 150 liters per minute, and dissolved oxygen was maintained at > 80% air saturation. Irrigation water had a pH of 7.5 to 7.8, hardness of 220 mg/L $CaCO_3$ and alkalinity of 180 mg/L as $CaCO_3$. The fish were acclimated on commercial salmonid diet (Nelson and Sons, Inc., Utah) for approximately one month after their arrival at UCD and were fully transitioned to experimental diet (0.3% body wt/day) by November 17, 2001. The two 6.1 m diameter tanks were cleaned weekly, with minimum disturbance to the fish.

Experimental Diet

Two diets were made with essential (control; *ca.* 1 μg/g) and elevated (treatment; *ca.* 34 μg/g) levels of Se (Table 2-1). Commercial salmonid diet (Nelson and Sons, Inc., Utah) was obtained as mash and pelleted with fish oil (10%) and selenized yeast (2.2%; Selenomax®, Ambi Inc.). Water was added prior to pelleting at 14% of the total diet. For the control diet, the selenized yeast mixture contained 1.3% selenized yeast and 98.7% tortula yeast. Only selenized yeast was added to the treatment diet. After pelleting, the diet was allowed to air dry on drying racks.

Sampling

On February 12, 2002 each female was biopsied for egg size and maturation. Fish were anesthetized in 50 mg/L MS-222 until a stage 4 to 5 anesthesia was achieved. A small incision was made in the abdomen to allow insertion of a catheter for the collection of eggs, which were placed into Ringers solution for analyses of egg diameter,

polarization index and response to progesterone exposure (as described below). Blood was collected from the caudal vasculature, using a 10 ml heparinized Vacutainer®, and centrifuged (4000 x g, 10 min) to separate the plasma, which was flash frozen in liquid nitrogen for protein isolation and Se analysis. Fish weight and fork length were also measured. Females were sampled repeatedly (approx every 4 weeks) until it was determined that a female was ready for spawning.

Egg Diameter, Polarization Index and Maturation Assay

Representative subsamples of vitellogenic eggs (n=15 from each fish) were collected by catheterization and the egg diameters were measured using a darkfield dissecting microscope with camera lucida, an image analyzing tablet, and a microcomputer interface (± 0.01 mm). The readiness of a specific female to spawn was determined by examining additional subsamples of collected eggs to calculate the polarization index (PI), and measure the capacity to undergo germinal vesicle breakdown (GVBD) in the presence of an in-vitro maturation-inducing steroid, progesterone (Dettlaff *et al.* 1993; Mims *et al.* 2002). Eggs were extracted by a catheter (Conte *et al.* 1988) and kept in a sturgeon Ringer solution (Dettlaff *et al.* 1993) with penicillin (0.03 g/L) and streptomycin (0.05 g/L). The incision for inserting the catheter is small (6-9 mm) and is closed using a single suture. Females are returned to the broodstock tank until spawning or further sampling. Domestic white sturgeon females have been sampled in this manner 3 to 5 times, every 4 to 6 weeks, and have been successfully induced to ovulate, producing eggs with high fertility and hatch rates (Van Eenennaam *et al.* 2004).

Subsamples of 15 eggs per female were used for PI determination. Eggs were boiled for 5 to 6 minutes, chilled on wet ice for 20 minutes, stored in 10% buffered formalin overnight, and bisected along the animal-vegetal axis using a thin, double-edged razor blade. The PI was calculated by measuring the distance of the germinal vesicle from the inner border of the egg chorion at the animal pole, and dividing this by the animal-vegetal egg diameter. These measurements were made using a darkfield dissecting microscope with camera lucida, an image-analyzing tablet, and a microcomputer interface (± 0.01 mm).

The PI alone provides a very good indication of female readiness to spawn, but it does not directly measure the capacity of the egg to mature. Thus, the catheterized eggs are also analyzed for their capacity to undergo GVBD in the presence of a maturation-inducing steroid. To determine the capacity of the egg to undergo GVBD, the *in vitro* egg maturation assay, as described in Dettlaff *et al.* (1993), was conducted in sturgeon Ringer solution at 16 °C for 16 hours. Fifteen eggs were incubated with 5 µg/mL progesterone and 15 were incubated without progesterone to serve as a control. Each treatment had two replicates. After incubation, the eggs are boiled, chilled on wet ice, stored in 10% buffered formalin, bisected and examined for GVBD. The criteria used to select females for induced ovulation were an egg PI of less than 0.10 and 100% GVBD response in the egg maturation assay.

Spawning Induction, Egg Fertilization and Embryo Incubation

The spawning, fertilization and incubation protocol followed Conte *et al.* (1988), with few modifications. The males received a single injection of 10 μg/kg body weight of mammalian GnRHa [D-Ala^6, Des-Gly^{10}]–LH-RH Ethylamide (Peninsula Laboratories), and the females received a priming injection of 2 μg/kg GnRHa, followed in 12 h with resolving injection of 18 μg/kg GnRHa. All injections were intramuscular and given underwater to minimize handling stress. Females were held in separate 3.7 m diameter tanks. Tank bottoms were routinely checked for released eggs beginning 16 h after the second injection. The ovulating female was placed onto a hooded stretcher and ovulated eggs were removed surgically (Conte *et al.* 1988). Females were anesthetized during surgery using MS-222 solution (100 mg/L), which was pumped across the gills using a submersible pump with a 5 cm diameter vinyl tube and a 100 L ice chest as a sump.

Two to three males were induced to spermiate at each spawning. Milt was collected from each male with a 60 ml plastic syringe and a 4 cm long vinyl catheter inserted into the urogenital pore (Conte *et al.* 1988). Sperm was evaluated for percent initial motility and the time to less than 10% motility under a compound microscope, using 5 μL of semen diluted immediately with 200 μL of hatchery water. Milt was only used if sperm had over 50% initial motility and duration to 10% motility was at least one minute and thirty seconds. Eggs were fertilized with 20 ml of pooled milt from 2 to 3 males, diluted 1:200 with hatchery water. After 3 min of fertilization, eggs were gently mixed in a water suspension of Fuller's Earth (100-200 mesh size, Sigma Chemical Co.) for 60 min to prevent egg adhesion. The number of ova collected from each female was

estimated volumetrically (egg count in five 5 ml subsamples). Approximately 1 Liter of fertilized eggs was placed into individual MacDonald hatching jars; and, jars were set-up to allow hatched larvae from individual females to flow into separate 1.2 m diameter circular tanks. Embryo incubation was at water temperatures 15 to 16 ºC.

Final Necropsy Sampling

Females were euthanized and necropsied following the extraction of ovulated eggs, or when determined to be unable to ovulate (Dettlaff *et al.* 1993). Euthanasia was performed using an overdose of MS 222 and severing the spinal cord. Samples of liver, kidney, muscle, spleen, eggs and post-ovulatory gonads were frozen on dry ice and stored at -80 ºC for Se analysis. Prior to euthanasia, blood was collected from the caudal vasculature using a 10 ml heparinized Vacutainer® and centrifuged (4000 x g, 10 min) to separate the plasma, which was flash frozen in liquid nitrogen and stored at -80 ºC for protein isolation and Se analysis. Eggs collected for egg yolk purification were flash frozen in liquid nitrogen and stored at -80 ºC. Developing embryos were sampled at stage 5 for fertilization success and stage 21 for neurulation success, as described in Dettlaff *et al.* (1993).

Vitellogenin Purification and Identification

Plasma

Plasma vitellogenin (VTG) was purified from estrogenized male white sturgeon plasma using anion exchange chromatography. To verify the identity of estrogen-induced VTG, the chromatographs of the male were compared before and after treatment

(Figure 2-2). Purification of VTG was accomplished as follows: 200 µL of plasma was diluted 1:10 into the running buffer (20 mM bis-tris-propane, 50 mM NaCl, pH 9.0) and then loaded onto the column containing the strong anion exchanger, 20 HQ, and separated using the BIOCAD Perfusion System $_{TM}$ (Perseptive Biosystems). The flow rate was maintained at 10 mL/min and 1.0 ml fractions were collected. The proteins absorbance and salt content of the eluent were measured at 280 nm and conductivity in µsiemens. Non-binding proteins were eluted by washing with 5 column volumes of running buffer (peak P1, Figure 2-3). Sequential removal of binding proteins (P2-P4) was accomplished by a linear gradient of salt (50-800 mM). VTG binds the strongest of the plasma proteins at pH 9.0 and is the last protein to elute from the column (Figure 2-3).

Plasma proteins other than VTG, indicated as P1-P4 (Figure 2-3), were pooled and indicated as "nonVTG" proteins in PAGE gels and Westerns. After pooling the fractions (VTG or nonVTG peaks), the pH was adjusted to 7.0 using 100 mM bis-tris-propane (pH 6.0), sodium azide (0.02% v/v), and protease inhibitor-Aprotinin (10 KIU/ml) was added. A 1.0 ml aliquot was collected from each pool, the cryoprotectant - glycerol added (50% v/v) and stored at –20 ºC. Freeze/thaw fracture of VTG and yolk proteins is a major contributor to degradation of yolk proteins and strongly affects ELISA results (personal observation by Kroll/Denslow, Hybridoma lab, University of Florida). Freeze/thaw fracture of yolk proteins is avoided by the addition of glycerol and storage at –20 ºC. At this temperature the solution remains a liquid. This aliquot would be used for PAGE and Western analysis. The total protein content of each pool was determined by Bradford analysis (Coomassie Plus$_{TM}$, Pierce) using bovine serum albumin as a standard.

VTG levels in the plasma were relatively low because the females used for this study were in late stages of vitellogenesis and were developing polarized eggs (Doroshov *et al.* 1997). Pooling several ion exchange runs was necessary to acquire enough VTG for analysis. In some cases further concentration using a 10,000 Da size-exclusion spin column (Microcon, Millipore) was necessary for VTG-PAGE analysis.

Plasma PAGE

In order to ascertain the purity and identity of the purified VTG, the plasma and purified protein pools were separated by electrophoresis on polyacrylamide gels (PAGE) and then transblotted to a membrane for Western analysis. A 4 to 12% gradient gel (NuPAGE$_{TM}$, Invitrogen) was used to capture all the white sturgeon plasma proteins. The samples run on a gel consisted of the following: 1) male negative control, 2) purified VTG positive control (VTG+, 1.0 µg/lane), 3) the plasma sample before purification, and 4) the pooled fractions after ion exchange purification (nonVTG proteins pool and VTG). Plasma was diluted 1:50, purified VTG and nonVTG pool was diluted to 0.05 µg/µL in a mixture of Laemmli sample buffer containing dithiothreitol as a reducing agent. The samples were heated to 95 ºC for 10 min and then loaded onto the gel. Ten microliters of each sample and a mixture of colored molecular weight markers (See Blue 2$_{TM}$, Invitrogen) were added to each lane. The amount 0.5 µg of each purified protein, VTG+, non-VTG and VTG proteins, were added to separate lanes. The gel was run for 35 min at 200 V. The gel was soaked for 2 to 4 hours in colloidal blue$_{TM}$ stain (Invitrogen) and destained using water. Each gel was scanned and labeled using the Adobe program. PAGE gels of pooled nonVTG plasma proteins showed bands at approximately 70, 62

and 28 kDa (Figure 2-4). The VTG proteins were identified using Western analysis, as described below.

Plasma Western

A gel identical to the PAGE gel was used for Western analysis. After running the PAGE gel, the gel was removed and transblotted to PVDF membrane (Invitrolon$_{TM}$, Invitrogen) for 3 hrs at 100 V (4 ºC) in buffer (10 mM MES, 10% MeOH, 0.01 % SDS, pH 6). The blot was blocked in blotto, which consisted of 5% instant milk in TBSTZ (20 mM tris, 150 mM NaCl, 0.05% Tween-20, 0.02% azide, pH 7.6) for 2 hrs. A highly specific monoclonal antibody (Mab), 1H12 (HL 1387) against gulf sturgeon (*Acipenser oncorynchus destoi*) VTG developed by the University of Florida Hybridoma lab (Kroll/ Denslow) was used to identify VTG on the blots. After blocking the membrane, the blot was incubated overnight in 0.1 ng/ml of the Mab at 4 ºC, washed twice with TBSTZ and then incubated for 2 hours in the secondary antibody, anti-mouse IgG (H&L)- alkaline phosphatase diluted 1:1000 in blotto. The blot was rewashed twice, and the color developed by adding the enzyme's substrate (1-Step$_{TM}$, Invitrogen). Development was stopped by washing with water. Each blot was dried, scanned, and labeled.

The anti-sturgeon VTG Mab is very sensitive and clearly recognizes white sturgeon purified positive control, gravid female plasma, and purified gravid female plasma VTG (Figure 2-4). It does not react with pooled nonVTG plasma proteins. The Mab recognizes 3 different bands of white sturgeon VTG. The VTG proteins are high molecular weight: 180, 75, and 64 kDa (Figure 2-4).

Vitellogenin ELISA

Plasma VTG concentrations were determined by sandwich ELISA using a white sturgeon polyclonal antibody, UF131, and gulf sturgeon monoclonal antibody, 1H2, developed by the Doroshov (UCD) and Denslow (UF) labs, respectively. The ELISA assay was run as follows. A 96-well ELISA plate (Nunc ^{TM}, Nalge) was precoated overnight at 4 ºC with 5 µg/mL of the Mab (1H2, HL 1387) in PBSZ (10 mM phosphate, 150 mM NaCl, 0.02% azide, pH 7.2). The plate was washed four times with PBSTZ (PBSZ with 0.05% tween) using a plate washer (Skan washer, Molecular Devices). The plate was blocked with 1% BSA in TBSTZ (20 mM tris, 150 mM NaCl, 0.05% tween-20, 0.02% azide, pH 7.4) for 2 hours at room temperature. The plate was re-washed with PBSZ before adding the samples and standards.

Unknown samples and controls (positive and negative) were diluted 1:100, 1:10,000, 1:100,000, and 1:1,000,000 in PBSZ with the protease inhibitor- Aprotinin (10 KIU/ml) and loaded onto the ELISA plate. A standard curve using purified white sturgeon VTG (0, 0.005, 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 µg/mL), negative controls (nonestrogenized male), and positive controls (vitellogenic female) to be used for intra and inter-assay determination were also added. Each sample and standard was loaded in triplicate (50 µL/well). After incubating overnight (4 ºC) in a humidified Tupperware® container, the plate was washed as previously described. The polyclonal antibody UF 131, diluted 1:1,000 in blocking buffer (1% BSA in TBSTZ) was added to each well (50 µL) and incubated for 2 hours at room temperature. After washing (4X PBSZT), an alkaline phosphatase conjugated detection antibody, goat-anti-rabbit IgG- $F(ab)_2$-AP (Pierce), diluted 1:1000 in blocking buffer was added and

incubated for 2 hours at room temperature. The plate was washed and then 1.0 mg/mL of the substrate, para-nitro phenyl phosphate (Sigma), in carbonate buffer (30 mM carbonate, 2 mM $MgCl_2$, and pH 9.6) was added to each well (100 µL/well). The plate was scanned at 405 nm using a reader (SpectraMax 384, Molecular Devices) and the data interpreted by the SoftMax Pro program.

A number of criteria were employed to maintain high quality data. The coefficient of variation between triplicate absorbance vales was $\leq$ 10%. Outliers were discarded as long as the CV $\leq$ 10% otherwise the sample was rerun. Absorbances at 405 nm greater than 1.5 were discarded. Inter and intra-assay variations (CV) were typically < 10% and < 5%, respectively as determined using positive and negative control samples. Figure 2-5 is a typical standard curve using purified white sturgeon VTG and the sandwich ELISA.

Egg Yolk Purification and Identification

Egg processing

Ten grams of frozen eggs were homogenized in 15.0 ml of gel filtration buffer (25 mM Trizma, 350 mM NaCl, 0.05% azide, pH 8.0) using a glass tissue grinder. The mixture was centrifuged for 30 min at 15,000 x g at 4 ºC. This resulted in 3 distinct layers: white fat, yellow aqueous and black pellet on the top, middle, and bottom of the tube, respectively. The middle layer was carefully removed using a pasture pipette and then recentrifuged to remove any remaining layers. The middle solution was filtered using a glass fiber filter (A/E, Pall) to remove any large particles. Azide (0.02%) was

added to this "crude egg yolk" preparation to prevent bacterial growth, and the solution was frozen (-20 ºC).

Three milliliters of the crude egg yolk was added to a gel filtration column (1.5 x 90 cm) containing Sepahrose-CL-6B (Pharmacia). This material is designed to separate proteins between 2,000,000 to 1,000 Daltons and has been cross-linked to improve flow rate. After loading the egg yolk solution, the flow was maintained at 0.3 mL/min and 6.0 ml fractions were collected every 20 min. The protein absorbance of each fraction was analyzed at 280 nm using UV 96-well plates (Greiner) and a plate reader (SpectraMax 384 Plus, Molecular Devices). After plotting the absorbance of each fraction three main peaks of different molecular weights were evident, indicated as E1 through E3 (Figure 2-6). Fractions within each peak were combined and the protease inhibitor-Aprotinin added (10 KIU/ml). The total protein of each pool was determined by Bradford analysis (Coomassie Plus, Pierce) using bovine serum albumin as a standard. A 1.0 ml aliquot of each pool to be used for PAGE and Western analysis was collected, glycerol added (50% v/v), and proteins were stored at –20 ºC.

Egg PAGE & Western

Polyacrylamide electrophoresis and Western analysis of egg yolk proteins (crude and purified) were identical to those described for plasma. Figure 2-7 shows typical gel and western of purified sturgeon egg proteins. The anti-sturgeon VTG Mab, 1H2, clearly recognizes the egg yolk proteins: crude preparation before purification, and peak E2. Peak E1 also reacts with the VTG antibody, but to a lesser degree. This may be due to slight contamination from peak E2 due to overlap and also the extremely high sensitivity

of the Mab. Peak E2 appears to be lipovitellin due to the cross-reactivity of the anti-VTG antibody. The Mab recognizes smaller sized proteins than plasma VTG. This may be a result of follicular processing and possible degradation of the proteins despite the addition of protease inhibitor. Peak E3 appears to be phosvitin since this peak contains small molecular mass proteins which do not bind to Coomassie Blue stain (Wallace and Morgan 1986). Evidently, this fraction is a phosphoprotein and stereotypical of phosvitins and phosvettes. Peak E1 seems to be immunoglobulin, based on its molecular mass (>600 kDa, Kroll 1990) and immunological studies on paddlefish (Acton *et al.* 1971) and adult white sturgeon (Adkison *et al.* 1996).

Analysis of Selenium in White Sturgeon Tissues by Hydride Generation ICP-AES

White sturgeon tissue samples were homogenized in millipure water using a hand blender and then freeze-dried. Approximately 300 mg of tissue was digested in a mixture of concentrated sulfuric, nitric and perchloric acids with a gradual temperature increase to 330 ^{o}C. The samples were then reduced by concentrated hydrochloric acid heated to 95 ^{o}C and measured as selenite by hydrogen generation inductively coupled plasma-atomic emission spectrometry (ICP-AES). The minimum detection limit was typically 5 ng/ml. Quality control measures included the use of standard reference materials (NRC DORM-2, DOLT-2, DOLT-3), spiked samples, duplication and blanks with every analytical run. All quality control measurements agreed with the target concentration within 20 percent.

Analysis of Selenium in Plasma and Isolated Proteins by Fluorometry

Plasma and isolated plasma and yolk proteins were analyzed for total Se using the method of Fan *et al.* (1998). VTG samples within the same exposure level were combined for Se analysis in order to obtain the minimum amount of material required. VTG sample combinations are shown in Table 2-2. Approximately 2 mg of freeze-dried proteins, or 100 µl plasma, were digested in a mixture of nitric and sulfuric acids and gradually heated to 130 ^{o}C. The samples were then reduced to selenite in 6 N HCl heated to 110 °C. The selenite was derivatized to piazselenol by the addition of 0.1% diaminonaphthalene (DAN) reagent (prepared by sonicating 2,3-diaminonaphthalene hydrochloride, Dojindo Laboratories (Japan), in 0.1 N HCl for 1 h, filtered through 0.45 µm cellulosic filter, and washed three times with excess cyclohexane) and extracted using 0.5 ml of cyclohexane. This mixture was incubated at 45 °C in the dark for 30 min, and then shaken to complete the extraction into the cyclohexane layer. Two hundred microliters of the cyclohexane layer was measured for the fluorescence intensity of the piazselenol derivative using a spectrofluorometer. The minimum detection limit was typically 1 ppb. Quality control measures included the use of spiked samples, duplication and blanks with every analytical run. Quality control measurements agreed with the target concentration within 15 percent.

Statistical Analysis

Morphologic measurements were compared between exposure groups using ANOVA and ANCOVA. Log_{10} transformations were used in regressions of body weight to fork length to account for allometric growth. The reproductive endpoints fecundity,

fertilization success and neurulation success were compared between exposure groups by the Wilcoxon Rank Sums Test. Comparisons of mean Se concentrations between two groups were performed using a least squares means Student's t-test, ANOVA or Wilcoxon's rank sum test. Se concentrations were compared across tissues and exposure groups using two-way ANOVA, nested by individual fish, with a Bonferroni-adjusted Students t-test (experiment-wise $\alpha = 0.05$; adjusted comparison-wise $\alpha = 0.00142$). Comparisons of Se concentrations between exposure groups at different sampling time points for plasma and isolated plasma proteins were performed using two-way ANOVA, nested for repeat measures, with Tukey's Honestly Significant Difference (HSD) comparisons test ($\alpha = 0.05$). Log_{10} transformations were used when necessary to meet the assumptions of parametric models. Nonparametric tests were used when the assumptions of parametric models could not be met. The relationships between Se concentrations in various tissues were examined using linear or nonlinear regressions. Correlation analysis was used when the variables could not be assumed to be functionally dependent. Weighted least squares regression was used when the dependent variable did not show constant variance. The weights used included the reciprocal of y and the reciprocal of y^2. The statistical software packages JMP (SAS Institute Inc.) and SigmaPlot (Systat Software, Inc.) were used for all computations.

One fish (treatment fish number 8) in the Se treatment group lost 2.75 kg (~10% body weight), had negative specific growth rate (- 0.05%), and tissue Se concentrations lower or similar to control group. Apparently, this female was not feeding due to avoidance of high Se feed, stress, or internal injury caused by transport to UCD or handling. This fish was excluded from all statistical analyses of Se data in this study.

Results

Gross Morphologic Characteristics

There were no differences in condition factor, fork length, or body weight between exposure groups at either the beginning or end of the study (ANOVA, $p > 0.18$; Table 2-3). Body weight to fork length regressions were not significantly different between exposure groups at either the beginning or end of the study (ANCOVA, $p = 0.69$ and 0.06 respectively). In addition, the specific growth rate (SGR) for the length of the study was not different between the two exposure groups (ANOVA, $p = 0.94$).

Adult Tissue Selenium

Comparisons within individual tissues showed that Se concentrations were significantly higher in Se-treated females (Student's t-test, $p < 0.0001$; Wilcoxon's Rank Sums, $\chi 2 < 0.0017$; Table 2-4). Se concentrations were also compared among all tissues and exposure groups (two-way nested ANOVA $p < 0.0001$; adjusted multiple comparisons, $\alpha = 0.05$; Figure 2-8). Again, treated females had significantly higher Se concentrations in liver, kidney, spleen, muscle, ovarian tissue, and eggs compared to control. In control fish, Se concentrations in kidney were greater than in muscle, ovarian tissues and liver, but similar to concentrations in spleen and eggs. Spleen of control fish contained higher levels of Se than did muscle. Se concentrations in ovarian (post-spawning) and liver tissues did not differ in control fish. In treatment fish, Se concentrations in kidney were greater than in muscle, ovarian tissues, eggs and liver, but not spleen. Se in treatment fish spleen was greater than in liver. The levels of Se in treatment fish muscle, ovarian tissue and eggs were very similar. All Se values reported

above are based on dry weight analyses. Percent moisture of each tissue type is listed in Table 2-5.

Plasma, Isolated Plasma VTG and nonVTG Plasma Protein Selenium

Treatment fish had significantly higher levels of Se in plasma, and isolated plasma proteins, than control fish (Student's t-test, $p < 0.02$; Wilcoxon's Rank Sums, $\chi 2 < 0.0017$; Table 2-6). Plasma was collected at two time points within the experiment, mid-experiment (88 days dietary exposure) and at spawning or necropsy (126 – 200 days dietary exposure). Se concentrations in plasma, isolated plasma VTG, or nonVTG plasma proteins did not differ between sampling time points within each exposure group, but were significantly different between exposure groups at each sampling time point (two-way nested ANOVAs, $p < 0.011$; Tukey HSD, $\alpha = 0.05$; Figures 2-9 – 2-11). In treatment fish, VTG contained higher Se concentrations than those found in nonVTG plasma proteins (two-way ANOVA, $p = 0.032$; Tukey HSD, $\alpha = 0.05$; Figure 2-12). Se concentrations of VTG and nonVTG plasma proteins were similar for control fish. Se values are reported as wet weight for plasma and dry weight for isolated plasma proteins.

Plasma VTG concentrations in control fish were 0.21 ± 0.053 mg/mL (8) and 0.1 ± 0.034 mg/mL (7) at mid- and end-experiment, respectively (ELISA). Treatment fish contained 0.28 ± 0.083 mg/mL (7) and 0.08 ± 0.022 mg/mL (8) plasma VTG at mid- and end-experiment, respectively. Data shown as mean mg VTG/ml plasma ± standard error (number of fish). Plasma VTG concentrations were greater during mid-experiment as compared to end-experiment, but were not different between control and treatment fish (two-way nested ANOVA, $p = 0.048$; Tukey HSD, $\alpha = 0.05$; Figure 2-13). Plasma VTG

concentrations are expected to decrease when sturgeon reach full oocyte maturation, or experience widespread atresia (e.g., fail to ovulate; Doroshov *et al.* 1997; Linares-Casenave *et al.* 2002).

Due to small sample sizes of isolated plasma VTG, samples from individual fish from the same exposure group were pooled prior to Se analysis (Table 2-2). Only one pooled VTG sample (n = 4 fish) was available for the final sampling point of treatment fish. One-sample t-tests were calculated between mean Se values and the single end data point for treatment VTG (26.29 μg/g Se; Figure 2-10). Both the mid- and end-experiment time points in the control group had mean VTG Se values significantly different from the end-experiment data point in the treatment group ($p < 0.02$). Within the treatment group, the mid-experiment mean VTG Se concentration was not different from the single end-experiment VTG data point ($p = 0.32$).

Yolk Protein Selenium

Se concentrations in isolated yolk proteins are presented in Table 2-7. Se was highly variable in both egg immunoglobulin and phosvitin. Only lipovitellin Se concentrations were found to be statistically different between exposure groups (Wilcoxon Rank Sums; $\chi^2 = 0.005$; Table 2-7). Phosvitin had higher Se concentrations than lipovitellin within the treatment group (Wilcoxon Rank Sums; $\chi^2 = 0.005$; Table 2-7). No other significant differences were observed. Se values for yolk proteins are reported as dry weight.

Relationships of Selenium Distribution in Tissues

We hypothesized that Se in the liver would become associated with the VTG molecule and transferred through the plasma to the eggs (Figure 2-1). Se concentrations in isolated plasma VTG increased exponentially with increasing Se in liver ($R^2 = 0.90$; $p < 0.0001$; Figure 2-14; Table 2-8). Se in plasma, ovarian tissue and eggs increased exponentially with increasing Se concentrations in VTG ($R^2 \geq 0.82$; $p \leq 0.0008$; Figure 2-15; Table 2-8). Se in the yolk lipovitellin, but not phosvitin or immunoglobulin, increased exponentially with increasing Se concentrations in VTG ($R^2 = 0.91$; $p < 0.0001$; Figure 2-16; Table 2-8). Exploring the relationship between Se in eggs and in isolated yolk proteins, we found that only Se levels in lipovitellin could be predicted by Se levels in whole eggs ($R^2 = 0.96$; $p < 0.0001$; Figure 2-17). Neither phosvitin nor immunoglobulin Se show significant relationships with whole egg Se.

We also explored the possibility of Se in nonVTG plasma proteins contributing to whole egg Se. Se concentrations in ovarian tissue, eggs, plasma, and nonVTG isolated plasma proteins increased linearly with increasing Se in liver (Figure 2-18; Table 2-9). Plasma Se showed a significant relationship with Se in isolated nonVTG plasma proteins ($p < 0.0001$; $R^2 = 0.82$ & 0.92 for mid- and end-experimental samples, respectively; Figure 2-19). Se concentrations in eggs increased linearly with Se in nonVTG proteins ($R^2 \geq 0.73$; $p \leq 0.0004$; Figure 2-20). In the yolk, lipovitellin Se concentrations were positively correlated with Se in nonVTG proteins collected mid-experiment ($r = 0.88$; $p = 0.0007$; logtransformed), and end-experiment ($r = 0.66$; $p < 0.0267$; logtransformed; Table 2-10). Se concentrations in phosvitin were positively correlated with Se in nonVTG proteins collected end-experiment ($r = 0.66$; $p < 0.0267$; logtransformed; Table

2-10), but not mid-experiment. There was no correlation between Se concentrations in nonVTG proteins and in egg immunoglobulin (Table 2-10). All Se values are reported as dry weight, except plasma.

Reproductive Characteristics

Five fishes from each exposure group were hormonally induced to ovulate. The remaining three fishes from each exposure group had either follicular atresia or underdeveloped eggs that did not reach maturation competence (Appendix B). Three out of the five control fish ovulated (the two that did not ovulate may have been injected too early; Table 2-11). Four out of the five treatment fish ovulated (although one only partially). The mean PI and percent GVBD was 0.09 and 100% for the ten induced fish, and 0.15 and 35.4% for the five individuals that were not induced to ovulate (one atretic female died mid-experiment due to technical error). Virgin (first reproductive cycle) females of cultured white sturgeon often do not respond to hormonal treatment as well as iteroparous (second or third cycle) females. Hence, the ovulation rate after hormone injection cannot serve as an endpoint in Se toxicity trials with a limited number of fish. Detailed information on spawning-related parameters is presented in Appendix B. No significant differences were found for fecundity (95 and 72 thousand, in control and treatment), fertilization rate (86 and 88%), and neurulation survival (60 and 47%) between ovulated fish from the two exposure groups (Wilcoxon; $p > 0.35$; Table 2-12). Hatching occurred in all egg batches, except of one female (in treatment), but hatching rates could not be properly measured under the condition of “in mass” egg incubation.

One fish (treatment fish 3) was excluded from the fecundity analysis due to incomplete ovulation.

Discussion

The bioaccumulation of Se in adult white sturgeon observed in this experiment concurs with several other studies showing the efficient uptake of Se in fish exposed to increased concentrations (Cleveland *et al.* 1993; Crane *et al.* 1992; Hamilton *et al.* 1990b; Hamilton *et al.* 2002b; Hilton *et al.* 1980). White sturgeon fed treatment diet (*ca.* 34 μg/g Se) had Se concentrations approximately 3 to 9 times greater than those fed control diet (*ca.* 1.42 μg/g Se) in all tissues analyzed, expect the yolk proteins phosvitin and egg immunoglobulin (Figures 2-8 – 2-12; Table 2-7). The kidney and spleen accumulated the greatest amounts of Se, most likely due to the high metabolic activities of these tissues (Diplock 1976; Hilton and Hodson 1983; Sorensen 1986). Relatively lower levels of Se were observed in liver, which is also highly metabolic. This may be indicative of the transport of Se from liver to developing eggs during vitellogenesis.

The average Se in isolated plasma VTG of treatment fish was 8 times higher than that of control fish, and approximated the concentration of the treatment diet (Figure 2-10). Furthermore, plasma VTG protein contained higher Se levels compared to the nonVTG plasma proteins in treatment fish (Figure 2-12). Se concentrations of VTG and nonVTG plasma proteins were similar for control fish. These results support a tentative conclusion of Kroll and Doroshov (1991), who hypothesized that VTG is a potential vehicle for the maternal transport of Se. Kroll and Doroshov (1991) found high, but variable, concentrations of Se in plasma and eggs from six white sturgeon females

captured in the Sacramento River. These sturgeon had Se concentrations ranging from 0.19 to 0.33 µg/ml in plasma, 3.06 to 29.26 µg/g in eggs, 2.78 to 4.11 µg/g in VTG, 0.76 to 6.70 µg/g in egg immunoglobulin, 3.14 to 11.22 µg/g in lipovitellin, and 3.17 to 90.31 µg/g in phosvitin (all values based on dry weight, except plasma). Se concentrations in ovulated eggs increased linearly with increasing Se in egg immunoglobulin and phosvitin (R^2 = 0.83 and 0.88, respectively; Kroll and Doroshov 1991).

These authors speculated that Se is most likely incorporated into the backbone of the VTG molecules by substituting for sulfur in the sulfur-containing amino acids, methionine, cysteine and cystine (two cysteine residues linked by a disulfide bond). Se is able to substitute for sulfur in biological molecules because these two elements have similar chemical properties (Diplock 1976; Stadtman 1974). White sturgeon VTG contains 1.49% cysteine and 2.92% methionine (based on total moles of amino acids; Bidwell and Carlson 1995). VTG can transport toxic levels of cationic metals (Ghosh and Thomas 1995; Lee 1993; Shackley *et al.* 1981) most likely by binding to phosphate residues. As a lipoglycophosphoprotein, VTG can transport lipophilic toxins such as DDT, dioxin and benzo(a)pyrene (Monteverdi and Di Giulio 2000; Plack *et al.* 1979; Ungerer and Thomas 1996). The work by Kroll and Doroshov (1991) was the first to indicate VTG as a potential vehicle for the maternal transport of Se, and the current study is the first to examine this exposure route experimentally.

The results of this study clearly indicate that Se accumulated in the liver becomes associated with VTG and is then transported to developing eggs with plasma proteins. As more Se is available in the liver, greater amounts of Se are associated with VTG (Figure 2-14). In fact, Se concentrations in VTG increased exponentially with increasing

Se concentrations in the livers of fish from this study ($R^2 = 0.90$; Figure 2-14). Se in plasma, ovarian tissue and eggs showed what appears to be an asymptotic exponential increase with increasing Se concentrations in VTG ($R^2 = 0.82 – 0.95$; Figure 2-15). This demonstrates the efficient and dose-dependent maternal transport of Se via VTG in white sturgeon.

The relationship between Se concentrations in VTG and in yolk proteins is not as clear (Figure 2-16). Only lipovitellin Se concentrations were found to have a significant relationship to Se in VTG and eggs ($R^2 = 0.91$ and 0.96, respectively; Figures 2-16 & 2-17), whereas Se concentrations of phosvitin and egg immunoglobulin were highly variable (CV ranged 0.44 to 1.10 in control). Kroll and Doroshov (1991) found higher levels of Se in phosvitin than in other yolk proteins. We observed the same trend in treatment fish of this study (Table 2-7); however, there were no statistical differences between mean Se concentrations in phosvitin and egg immunoglobulin across exposure groups. It is possible that data from a greater number of individuals would have decreased the variation observed in phosvitin and egg immunoglobulin Se concentrations. The impurity of the peak E1 and E3 fractions could also contribute to a high variation of Se concentrations in immunoglobulin and phosvitin. Interestingly, Hiramatsu *et al.* (2002) reported a relatively high proportion of methionine (~2.5%) in lipovitellin purified from yolk proteins of fertile sturgeon hybrid *Huso huso x Acipenser ruthenus*, but no detectable level of this amino acid in the purified phosvitin fraction.

Several possible mechanisms exist for the incorporation of Se into the VTG molecule. Se is incorporated into proteins as a result of genetic translation (reviewed by Gromer *et al.* 2005; Suzuki 2005a). A specific genetic codon prescribes the

incorporation of selenocysteine (SeCys) into proteins; however, selenomethionine (SeMet) is indiscriminately substituted for the methionine codon in genetic translation. As noted above, white sturgeon VTG consists of 1.49% cysteine and 2.92% methionine (based on total moles of amino acids; Bidwell and Carlson 1995). The amount of SeMet that is indiscriminately incorporated into proteins increases with increasing availability of SeMet in tissues (Schrauzer 2000). Similarly, when increased Se is available compared to sulfur, SeCys can substitute for cysteine in protein synthesis (Kramer and Ames 1988; Müller *et al.* 1994). The results of this study support the hypothesis that as Se becomes more available in the liver, there is an increase in the substitution of Se amino acids for sulfur amino acids during protein synthesis.

Considerable amounts of Se were also found to be associated with nonVTG plasma proteins; however, the roles of these proteins in egg development are not well known. We observed a significant linear relationship between Se concentrations in liver and nonVTG plasma proteins ($R^2 = 0.65$; Figure 2-18; Table 2-9). The Se concentrations in eggs increased linearly with increasing Se concentrations in nonVTG plasma proteins ($R^2 = 0.80$ and 0.73 for mid- and end-experiment samples, respectively; Figure 2-20), which may simply reflect the effect of increasing liver Se. In addition, Se concentrations in the yolk proteins lipovitellin and phosvitin were positively correlated with those of nonVTG plasma proteins (Table 2-10). This may suggest that at least some of the nonVTG plasma proteins contribute Se to developing eggs.

PAGE gels of the pooled nonVTG plasma proteins show strong bands at approximately 70, 62 and 28 kDa (Figure 2-4). The identification of these proteins is unknown; however, several known plasma proteins associated with Se correspond with

these general molecular mass ranges. In particular, plasma albumin (68 kDa in humans) is a very abundant plasma protein that binds with selenide in the bloodstream (Quinlan *et al.* 2005; Shiobara and Suzuki 1998). In addition, several selenoproteins have been identified in vertebrates (reviewed by Gromer *et al.* 2005; Suzuki 2005a). Most importantly, the presence of excess cellular Se can significantly increase the production of selenoproteins (Fletcher *et al.* 2000; Helmy *et al.* 2000; Jameson *et al.* 2002). In humans, the predominant plasma selenoprotein is selenoprotein P (Sel P; 42 kDa), which provides more than half of the total Se in plasma (Mostert 2000; Peri S. *et al.* 2003). Plasma Sel P is mainly synthesized in the liver and is the only selenoprotein known to incorporate multiple SeCys residues (Hill *et al.* 1991; Motchnik and Tappel 1990; Schweizer *et al.* 2005). Sel P in zebrafish (*Danio rerio*) contains seventeen selenocysteines (~ 50 kDa; Kryukov and Gladyshev 2000; Tujebajeva *et al.* 2000). The apparent function of Sel P is to transport Se from the liver to other organs (Burk *et al.* 1991; Hill *et al.* 2003; Saito and Takahashi 2002). Additionally, yolk cells of zebrafish embryos exhibit Sel P gene expression (Thissea *et al.* 2003; Tujebajeva *et al.* 2000).

Other selenoproteins present in plasma may include the enzymes, glutathione peroxidases (~ 25 kDa), thyroxide deiodinase (~ 30 kDa), and thioredoxin reductases (~ 55 kDa; Gromer *et al.* 2005; Kryukov and Gladyshev 2000; Tujebajeva *et al.* 2000). To date, none of the above selenoproteins have been characterized in white sturgeon. Further study is needed to understand the role of these unidentified proteins in Se maternal transport.

Several field and laboratory studies have observed Se maternal transport in fish, and consequent developmental toxicity. Gillespie and Baumann (1986) collected and

spawned female bluegills (*Lepomis macrochirus*) from a high-Se reservoir with egg Se concentrations of 12 to 55 µg/g. The resulting larvae exhibited edema, skeletal deformities, and died. Woock *et al.* (1987) found similar results. Schultz and Hermanutz (1990) experimentally demonstrated the efficient maternal transport of Se in fathead minnow (*Pimephales promelas*) raised in experimental streams. These authors showed that embryos collected from Se-exposed streams contained *ca.* 19.5 µg/g Se (converted from wet weight using 80% moisture) and developed into larvae with edema and skeletal deformities. Hermanutz *et al.*, (1992) observed reduced hatching, edema, skeletal deformities and hemorrhaging in progeny of bluegills held in experimental streams treated with 10 µg/L Se. Hamilton *et al.* (2002a) showed that razorback suckers (*Xyrauchen texanus*) in the seleniferous Colorado River can transfer significant amounts of Se to their eggs (36 – 60 µg/g at two sites). These authors determined that a Se burden of 20 µg/g or more in razorback sucker eggs represented a high level of hazard to this endangered species. Holm *et al.* (2005) found 41.85 and 31.85 µg/g Se (converted from wet weight using 80% moisture) in the eggs of rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*) collected from seleniferous areas in Canada. These eggs produced larvae with edema as well as craniofacial and skeletal deformities.

In the present study, white sturgeon fed a diet containing *ca.* 34 µg/g Se were found to contain *ca.* 11, 10 and 12 Se µg/g (dw) in muscle, liver and eggs, respectively. The treatment diet contained higher levels of Se than are typically found in the San Francisco Bay-Delta food web. The ubiquitous bivalve *Potamocorbula amurensis* is a primary food source for white sturgeon from this area and contain an average Se level of 15 µg/g (dry wt; Linville *et al.* 2002; Urquhart and Regalado 1991). However, the above

Se concentrations in muscle, liver and eggs are similar to those of wild white sturgeon captured from San Francisco Bay-Delta. White sturgeon sampled from this area between 1986 and 1990 contained Se at concentrations ranging from 9 to 30 μg/g in liver (n= 52) and 7 to 15 μg/g in muscle (n= 99; dry wt; Urquhart and Regalado 1991; White *et al.* 1988). Stewart *et al*. (2004) reported a mean liver Se of 24 μg/g (n=15; dry wt.) in white sturgeon collected from northern San Francisco Bay. Out of six sturgeon females sampled in the San Francisco Bay-Delta region in the 1990's, one contained eggs with 3 μg/g Se, four had eggs ranging from 8 to 12 μg/g Se and one female contained eggs with 29 μg/g Se (Kroll and Doroshov 1991). Recently, three white sturgeon captured from this region were found to have 7 to 20 μg/g Se in ovaries containing developing eggs (Doroshov Lab, UCD; unpublished data). The actual Se concentrations in the experimental animals are more important here than the relevance of the dietary treatment level since we are exploring the transport of accumulated Se from the liver to the developing eggs. Se concentrations in fish receiving treatment diet reached levels that have been associated with reproductive failure in other fish species (Garrett and Inmann 1984; Hermanutz *et al.* 1992). Lemly (2002) proposed thresholds of Se toxic effects for freshwater and anadromous fish at 8 μg/g in muscle, 12 μg/g in liver, and 10 μg/g in ovary or eggs. Mean muscle and ovary Se concentrations in treatment fish surpassed these thresholds, while Se in liver approached the suggested threshold of 12 μg/g. The reproductive endpoints of fecundity, fertilization rate and neurulation rate were not different between control and treatment fish of this study. The lack of impact on fecundity and fertilization is not unusual with Se toxicity (e.g., Coyle *et al.* 1993; Gillespie and Baumann 1986; Woock *et al.* 1987), since most organic Se is likely stored

in the yolk. However, as shown in the next chapter, embryos of the treatment fish from this experiment showed significant developmental toxicity.

In conclusion, this is the first work to experimentally demonstrate that vitellogenesis provides a significant, dose-dependent, mechanism of Se maternal transport in a fish species. White sturgeon is especially vulnerable to Se maternal transport due to a prolonged (two year) period of vitellogenesis and yolk deposition (Doroshov 1985; Doroshov *et al.* 1997). In contrast to other fish, sturgeon store and use their yolk in the form of intracellular crystalline platelets utilized throughout early development, including the embryonic and larval periods (Dettlaff *et al.* 1993). The secondary degradation of yolk proteins occurring at egg maturation (Hiramatsu *et al.* 2006; LaFleur *et al.* 2005; Romano *et al.* 2004) has not been studied in sturgeon; however, it appears that incorporated Se is conserved in the eggs through the vitellogenic phase of oogenesis until spawning. The results of our study, along with the field data described above, demonstrate that exposure of maturing females to a diet moderately high in Se may present a threat to the reproduction of white sturgeon residing in the San Francisco Bay-Delta region. This threat becomes more prominent as we explore Se-induced developmental toxicity in white sturgeon in the following chapter.

Se content of experimental diets

Diet	Target Se Content (µg/g, dry wt)	Measured Total Se (µg/g, dry wt)
Control	1	1.42 ± 0.03 (6)
Treatment	35	34.04 ± 0.82 (5)

Table 2-1. Selenium averages ± standard errors (n) are shown. Selenium was added to the diet as selenized yeast (Selenomax®, Ambi Inc.).

Combined VTG protein samples

	Days of Dietary Exposure	Fish Number	% of Combined Sample	Combined Sample ID	Time Point
Control	88	C1	45.2%		
	88	C2	26.5%		
	88	C3	28.3%	C1 - C3	Mid
	88	C4	100.0%	C4	Mid
	88	C5	27.0%		
	88	C6	32.1%		
	88	C7	30.0%		
	88	C8	10.9%	C5 - C8	Mid
	126	C1	72.7%		
	126	C2	27.3%	C1 & C2	End
	140	C3	35.3%		
	154	C4	29.4%		
	168	C5	5.0%		
	200	C6	15.1%		
	200	C7	15.1%	C3 - C7	End
Treatment	88	T1	50.8%		
	88	T2	47.2%		
	88	T3	2.0%	T1 - T3	Mid
	88	T4	54.7%		
	88	T5	14.6%		
	88	T6	30.7%	T4 - T6	Mid
	88	T6	100.0%	T7	Mid
	126	T1	58.4%		
	140	T2	22.2%		
	154	T3	3.1%		
	168	T4	16.3%	T1 - T4	End

Table 2-2. Sample combinations for analysis of VTG at mid- (88 days) and end-experiment (126 – 200 days). The percent contribution from each fish is shown. End sample T5 - T7 was excluded since it included VTG from fish T8, which was not included in Se analyses due to food avoidance (as described in the *Statistical Analyses* section).

Morphological comparisons between exposure groups

	Start of Study			End of Study		
ANCOVA	F	p	R^2	F	p	R^2
Length to Weight Regression *(log transformed)*	0.17	0.69	0.83	4.36	0.06	0.78
ANOVA	F	p		F	p	
Weight *(log transformed)*	1.98	0.18		1.49	0.24	
Fork Length *(log transformed)*	1.46	0.25		0.76	0.40	
Condition Factor	0.001	0.97		0.03	0.86	
SGR				0.006	0.94	

Table 2-3. Morphological characteristics were compared between control and treatment groups at both the beginning and end of the study. None of the measured characteristics were shown to be statistically different.

Se in tissues of adult white sturgeon fed either control or treatment diets for approximately six months

Tissue	Control	Treatment
Liver	**1.52 ± 0.23**	**9.63 ± 0.89***
Kidney	**4.64 ± 0.70**	**33.37 ± 3.04***
Spleen	**2.38 ± 0.13**	**20.57 ± 2.84†**
Muscle	**1.23 ± 0.15 (6)**	**11.15 ± 0.78***
Ovarian Tissue	**1.41 ± 0.19**	**9.6 ± 1.42***
Eggs	**2.2 ± 0.18 (5)**	**12.43 ± 1.70***

Table 2-4. Se levels measured in tissues of white sturgeon fed either control (*ca.* 1.42 µg/g Se) or treatment (*ca.* 34 µg/g Se) diets for approximately six months. Data displayed as mean Se µg/g, dw ± standard error. Sample size is 7 unless indicated in parentheses. Data are graphically displayed in Figure 2-8. *Significantly different value compared to control as shown by Students t-test on lognormal data ($p < 0.0001$). †Significantly different value compared to control as shown by Wilcoxon rank sum test ($\chi 2 < 0.0017$).

	Percent Moisture
Plasma	**94.8 ± 0.4**
Kidney	**83.7 ± 4.3**
Spleen	**83.5 ± 2.3**
Muscle	**75.0 ± 7.3**
Ovarian	**73.8 ± 13.4**
Eggs	**63.7 ± 4.1**
Liver	**53.6 ± 9.0**

Table 2-5. Percent moisture of adult white sturgeon tissues *(n=7 for plasma, n=3 for all other tissues).*

Se in plasma and isolated plasma proteins of adult white sturgeon fed either control or treatment diets for approximately six months

Tissue	Control	Treatment
Plasma[1] (µg/mL)	**0.21 ± 0.01 (8)**	**1.28 ± 0.10****
Plasma[2] (µg/mL)	**0.27 ± 0.05**	**1.25 ± 0.15****
NonVTG[1] Plasma Proteins	**7.27 ± 0.30**	**22.21 ± 3.38[†]**
NonVTG[2] Plasma Proteins	**9.1 ± 1.47**	**24.89 ± 3.43****
VTG[§1]	**4.29 ± 0.61 (3)**	**37.03 ± 8.18 (3)***
VTG[§2]	**4.31 ± 1.19 (2)**	**26.29 (1)[‡]**

Table 2-6. Se levels measured in plasma and isolated plasma proteins of white sturgeon fed either control (*ca.* 1.42 µg/g Se) or treatment (*ca.* 34 µg/g Se) diets for approximately six months. Data displayed as mean Se µg/g, dw ± standard error, except plasma, which is presented as Se µg/ml. Sample size is 7 unless indicated in parentheses. Data displayed graphically in Figures 2-9 – 2-11.

[1]Sampled after 88 days of exposure

[2]Sampled at spawning or necropsy, after 126 - 200 days of exposure

[§]Several VTG samples were pooled within treatments to provide enough sample material (Table 2-2)

*Significantly different value compared to control as shown by Students t-test on lognormal data (**$p < 0.0001$ or *$p < 0.002$)

[†]Significantly different value compared to control as shown by Wilcoxon rank sum test ($\chi 2 < 0.0017$)

[‡]Difference shown by one-sample t-test comparing control mean to the one treatment value ($p < 0.02$)

Se in isolated yolk proteins of adult white sturgeon fed either control or treatment diets for approximately six months

Tissue	Control	Treatment
Egg Immunoglobulin	11.95 ± 4.61 (3)	18.43 ± 3.28 (6)
Lipovitellin	5.14 ± 0.64 (5)	14.03 ± 1.62 (7)[†§]
Phosvitin	20.49 ± 9.74 (4)	42.59 ± 17.71 (7)[§]

Table 2-7. Se concentrations in yolk proteins isolated from eggs of adult white sturgeon following exposure to either control (*ca.* 1.42 µg/g Se) or treatment (*ca.* 34 µg/g Se) diet for 126 – 200 days. Data displayed as mean Se µg/g, dw ± standard error (n). [†]Only Se concentrations in lipovitellin were found to be different between exposure groups (Wilcoxon Rank Sums; $\chi^2 = 0.005$). [§]Treatment phosvitin was shown to have higher levels of Se than treatment lipovitellin (Wilcoxon Rank Sums; $\chi^2 = 0.005$). No other significant differences were observed.

Se in combined VTG samples compared to combined tissue and yolk protein samples
Data presented as mean Se µg/g, dw ± standard error, except plasma, which is presented as Se µg/ml

Control

Fish Number(s)	Time Point	VTG	Liver	Egg	Ovarian Tissue	Plasma	Phosvitin	Lipovitellin	Egg Immunoglobin
C1 – C3	Mid	3.57 ± 0.17 (3)	1.13 ± 0.10 (3)	2.23 ± 0.12 (3)	0.99 ± 0.27 (3)	0.19 ± 0.01 (3)	14.18 ± 10.49 (3)	5.96 ± 0.39 (3)	17.36 (1)
C4	Mid	3.8 ± 0.00 (1)	0.8 (1)	1.61 (1)	1.65 (1)	0.2 (1)		4.95 (1)	15.71 (1)
C5 - C8	Mid	5.51 ± 0.00 (4)	2.16 ± 0.05 (3)	2.68 (1)	1.75 ± 0.19 (3)	0.24 ± 0.01 (4)	39.41 (1)	2.88 (1)	2.79 (1)
C1 - C2	End	5.5 ± 0.26 (2)	1.03 ± 0.04 (2)	2.12 ± 0.03 (2)	0.74 ± 0.17 (2)	0.21 ± 0.04 (2)	3.70 ± 0.56 (2)	5.84 ± 0.65 (2)	
C3 - C7	End	3.12 ± 0.23 (5)	1.72 ± 0.28 (5)	2.25 ± 0.33 (3)	1.68 ± 0.11 (5)	0.3 ± 0.07 (5)	37.28 ± 2.13 (2)	4.67 ± 0.97 (3)	11.95 ± 4.61 (3)

Treatment

Fish Number(s)	Time Point	VTG	Liver	Egg	Ovarian Tissue	Plasma	Phosvitin	Lipovitellin	Egg Immunoglobin
T1 - T3	Mid	49.34 ± 1.37 (3)	10.44 ± 0.88 (3)	13.04 ± 3.86 (3)	8.49 ± 2.37 (3)	1.39 ± 0.25 (3)	20.86 ± 2.17 (3)	14.79 ± 3.52 (3)	18.72 ± 2.48 (3)
T4 - T6	Mid	21.53 ± 0.55 (3)	8.17 ± 1.64 (3)	10.94 ± 1.66 (3)	9.11 ± 1.99 (3)	1.19 ± 0.04 (3)	73.34 ± 36.88 (3)	12.07 ± 1.24 (3)	20.15 ± 11.44 (2)
T7	Mid	40.21 ± 2.06 (1)	11.6 (1)	15.1 (1)	14.4 (1)	1.22 (1)	15.55 (1)	17.63 (1)	14.09 (1)
T1 - T4	End	26.29 ± 1.52 (4)	9.77 ± 0.91 (4)	12.4 ± 2.80 (4)	7.97 ± 1.75 (4)	1.42 ± 0.20 (4)	23.25 ± 2.84 (4)	14.12 ± 2.58 (4)	16.22 ± 3.05 (4)

Table 2-8. Due to small sample sizes, isolated VTG protein samples were pooled prior to Se analysis (Table 2-2). Here we present Se in pooled VTG samples compared to the corresponding combinations of liver, eggs, ovarian tissue, plasma and isolated egg yolk proteins. Data displayed as mean Se µg/g, dw ± standard error (number of experimental animals contributing to the average), except plasma, which is presented as Se µg/ml. Mid samples were taken after 88 days of dietary exposure. End samples were taken after 126 – 200 days of dietary exposure.

Variables	Model	R^2	F stat	p value	n=	Equation
Liver v Eggs	Linear	0.82	46.25	<0.0001	12	y = 1.21x + 0.71
Liver v Ovarian	Linear	0.83	58.00	<0.0001	14	y = 1.00x - 0.08
Liver v Plasma[1]	Weighted Linear	0.87	83.10	<0.0001	14	y = 0.12x + 0.05
Liver v Plasma[2]	Linear	0.76	39.01	<0.0001	14	y = 0.11x + 0.13
Liver v nonVTG[1]	Linear	0.65	20.54	0.0009	13	y = 1.81x + 4.56
Liver v nonVTG[2]	Linear	0.65	21.98	0.0005	14	y = 1.88x + 6.49

Table 2-9. Statistical parameters for the regressions of Se concentrations in ovarian tissue, eggs, plasma and nonVTG isolated plasma proteins as a function of Se concentrations in liver (Figure 2-18). [1]Sampled after 88 days of exposure. [2]Sampled at spawning or necropsy, after 126 - 200 days of exposure. Each regression is statistically significant.

Se in yolk proteins correlated to Se in nonVTG

	nonVTG[1] Proteins	nonVTG[2] Proteins
Liver	**0.86**	**0.89**
Lipovitellin	**0.88**	**0.66**
Phosvitin	0.18	**0.66**
Immunoglobulin	0.62	0.22
Egg	**0.91**	**0.88**

Table 2-10. Pearson correlation coefficients for Se in plasma nonVTG proteins correlated to Se in liver, yolk proteins, and eggs (logtransformed data). Significant Pearson coefficients are presented in bold ($p < 0.05$). Se in nonVTG was positively correlated with Se in liver, lipovitellin, phosvitin, and whole eggs. [1]Collected after 88 days of exposure. [2]Collected after 126 - 200 days of exposure.

White sturgeon spawning data

Fish Number	Date Spawned/ Necropsied	Days of Dietary Exposure	Percent Ovulation	Estimated Fecundity (number of eggs)	Egg Fertilization Rate (%)	Percent Neurulated Embryos
C1	3/22/2002	126	None			
C2	3/22/2002	126	None			
C3	4/5/2002	140	100	73,440	88	36
C4	4/19/2002	154	100	137,243	84	88
C5	5/3/2002	168	100	73,080	86	56
C6	6/4/2002	200	Atretic			
C7	6/4/2002	200	Atretic			
T1	3/22/2002	126	100	101,238	92	76
T2	4/5/2002	140	100	84,830	88	74
T3	4/19/2002	154	Partial (40-50%)	31,968	94	36
T4	5/3/2002	168	100	66,660	76	0
T5	5/17/2002	182	None			
T6	5/17/2002	182	Eggs not polarized			
T7	6/4/2002	200	Eggs not polarized			
T8	6/4/2002	200	Eggs not polarized			

*Fish C8 died mid-experiment due to technical error (escaped tank through a rip in the tank covering).

Table 2-11. Results of spawning white sturgeon fed either control (*ca.* 1.42 μg/g Se) or treatment (*ca.* 34 μg/g Se) diets for approximately six months. More detailed spawning data are presented in Appendix B.

Reproductive performance of sturgeon females in control and treatment groups

Exposure	Estimated Fecundity (number of eggs)	Egg Fertilization Rate (%)	Percent Neurulated Embryos
Control	94,588 ± 21,328 (3)	86 ± 1 (3)	60 ± 15 (3)
Treatment	84,243 ± 9,986 (3)	88 ± 4 (4)	47 ± 18 (4)

Table 2-12. Fecundity, fertilization success and neurulation rates in control and treatment groups. Data presented as mean ± standard error (n). No significant differences were found between control and treatment groups for any of the three parameters (Wilcoxon; $p > 0.35$).

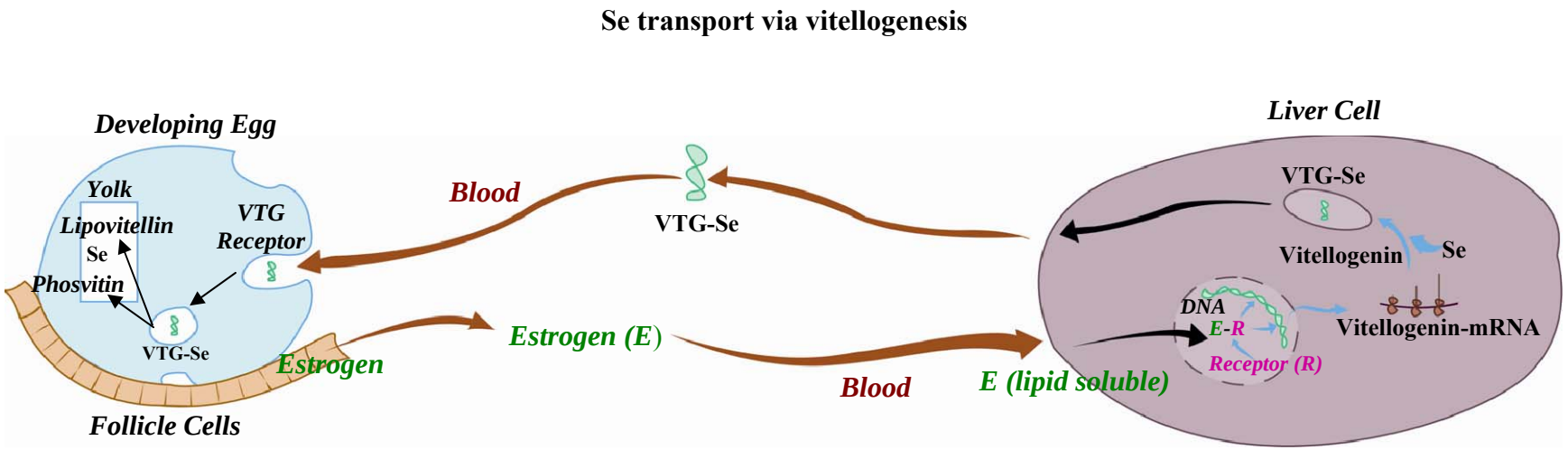


Figure 2-1. Hypothesized mechanism of the maternal transport of Se to offspring. During vitellogenesis (yolk deposition), estrogen is produced in egg follicles and transported to the liver via blood. Estrogen signals the liver to produce the yolk protein precursor, vitellogenin (VTG). Se in the liver may be incorporated into the VTG molecule, which is transported to the egg via blood and taken up by the VTG-specific receptor in the egg membrane. Inside the egg, VTG cleaves into the yolk proteins lipovitellin and phosvitin, and Se is stored in the yolk until metabolized during the embryonic and larval stages. Figure modified from Mommsen & Walsh (1988).

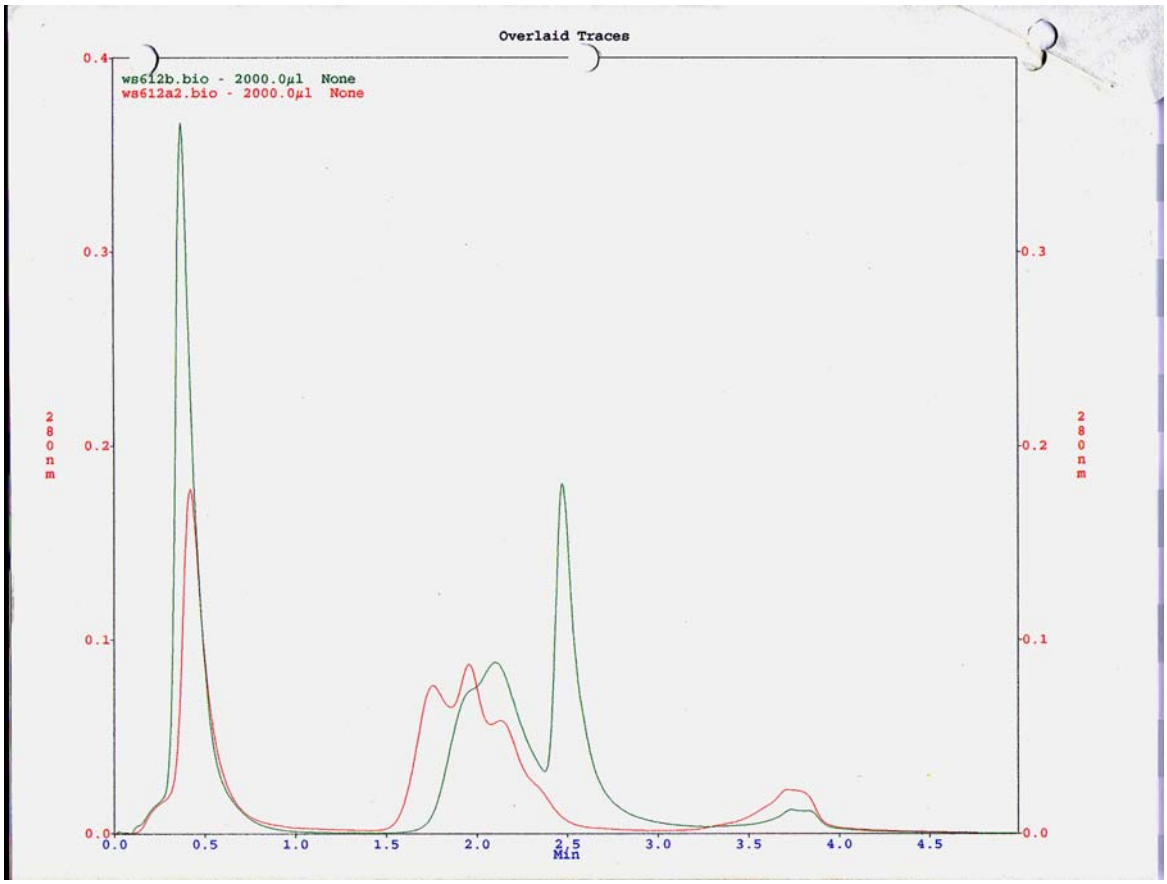


Figure 2-2. Anion exchange chromatograph of white sturgeon male before (red line) and after (green line) estrogen treatment.

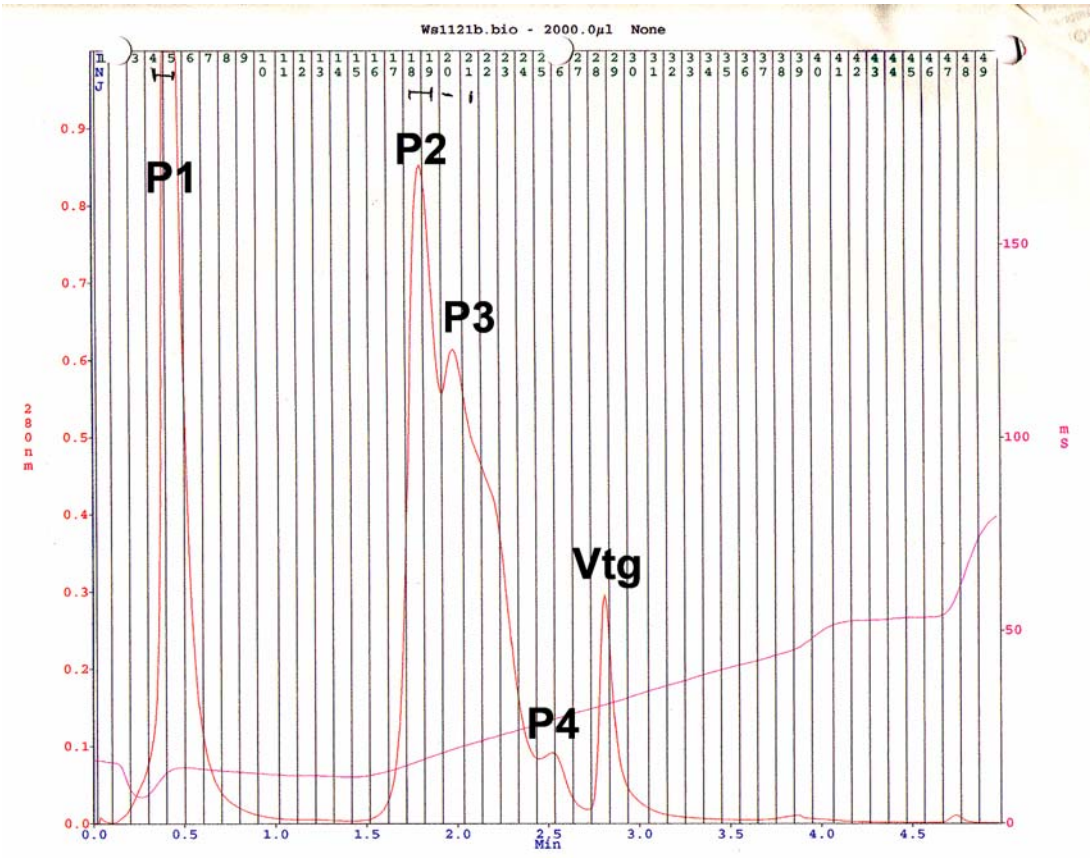
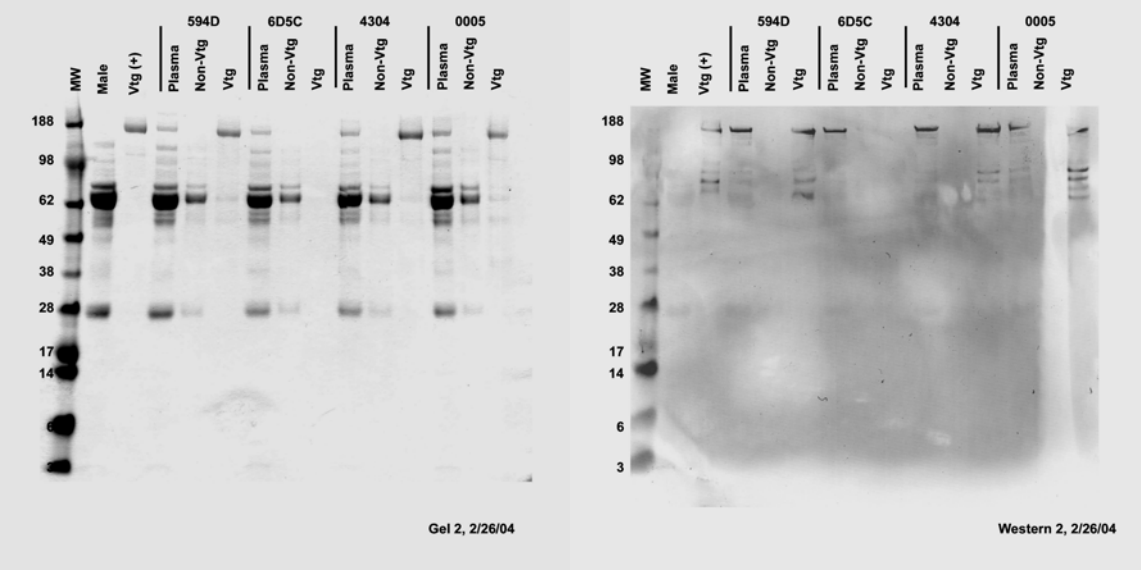


Figure 2-3. Anion exchange chromatograph of plasma proteins from vitellogenic white sturgeon female used for this study. P1, P2, P3, & P4 were pooled and indicated as "nonVTG" peaks. VTG is the vitellogenin peak.



PAGE **Western**

Figure 2-4. PAGE & Western of white sturgeon plasma proteins. The first lane contains molecular mass markers; the second lane contains plasma of nonestrogenized males; the third lane contains purified VTG. The next four groupings are individual females (coded by numbers), with lanes showing plasma, nonVTG pooled proteins, and isolated VTG protein. The PAGE gel of the pooled nonVTG proteins shows a strong band at 62 kDa and two weak bands at 70 and 28 kDa; none of which reacted with anti-VTG antibody in Western. The Western identifies three different bands of white sturgeon VTG: a strong band at 180 kDa and two weak bands at 75and 64 kDa. Fish 6D5C had VTG in plasma but VTG was not detectable in eluted fractions.

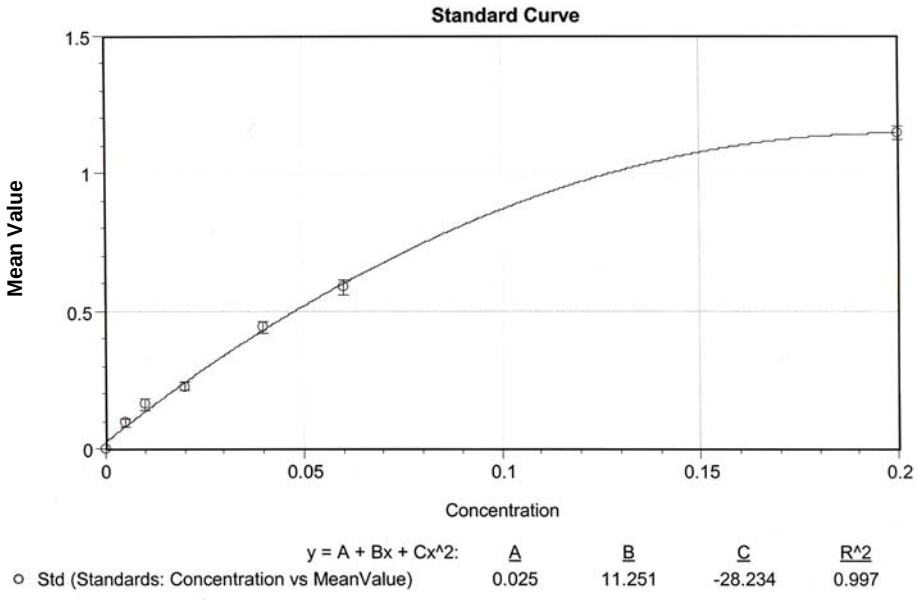


Figure 2-5. VTG ELISA Standard Curve. The x-axis shows VTG concentration (µg/ml) while the y-axis shows absorbance at 405 nm.

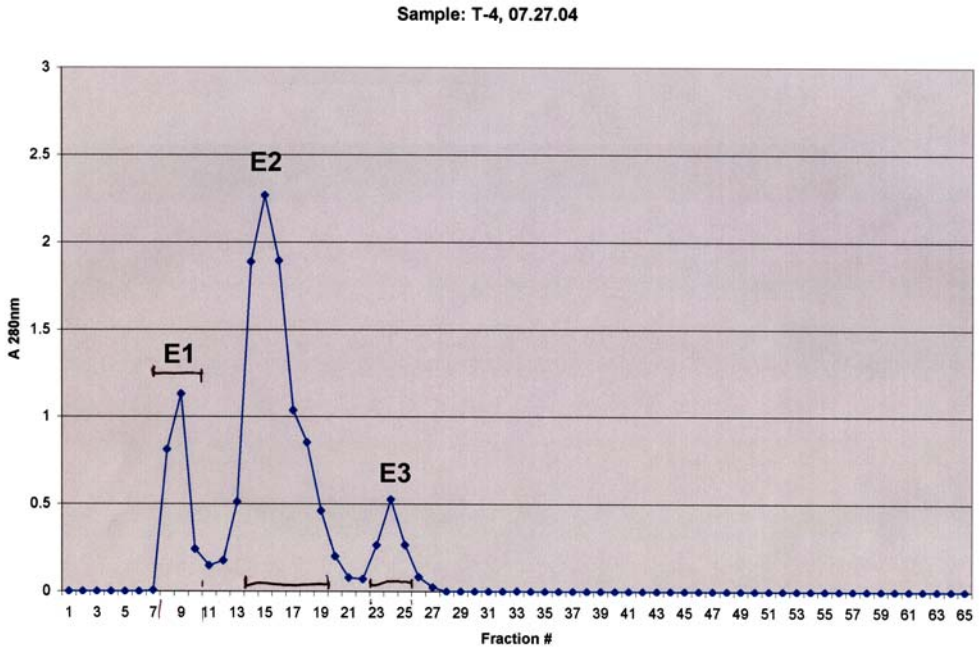
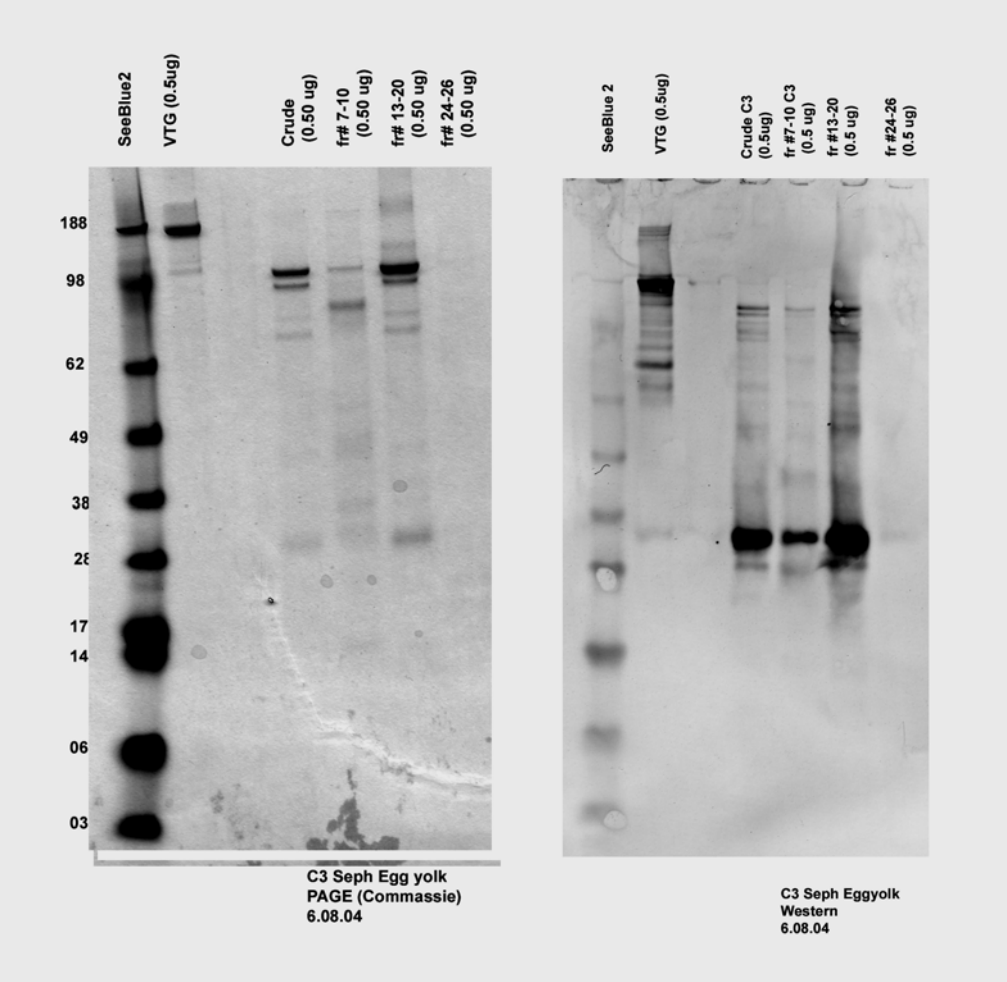


Figure 2-6. Gel filtration chromatograph of sturgeon egg yolk. Peaks E1, E2, and E3 were identified as egg Immunoglobulin (Ig), Lipovitellin (Lv), and Phosvitin (Pv). Horizontal bars show collected fractions.



PAGE Western

Figure 2-7. PAGE and Western gels of crude and purified egg yolk proteins of white sturgeon. The first two lanes are molecular markers and purified VTG. The next four lanes are crude yolk protein, and pooled fractions from gel filtration corresponding to peaks E1, E2, and E3. Peak E3 (Phosvitin) does not have affinity to Coumassie Blue stain.

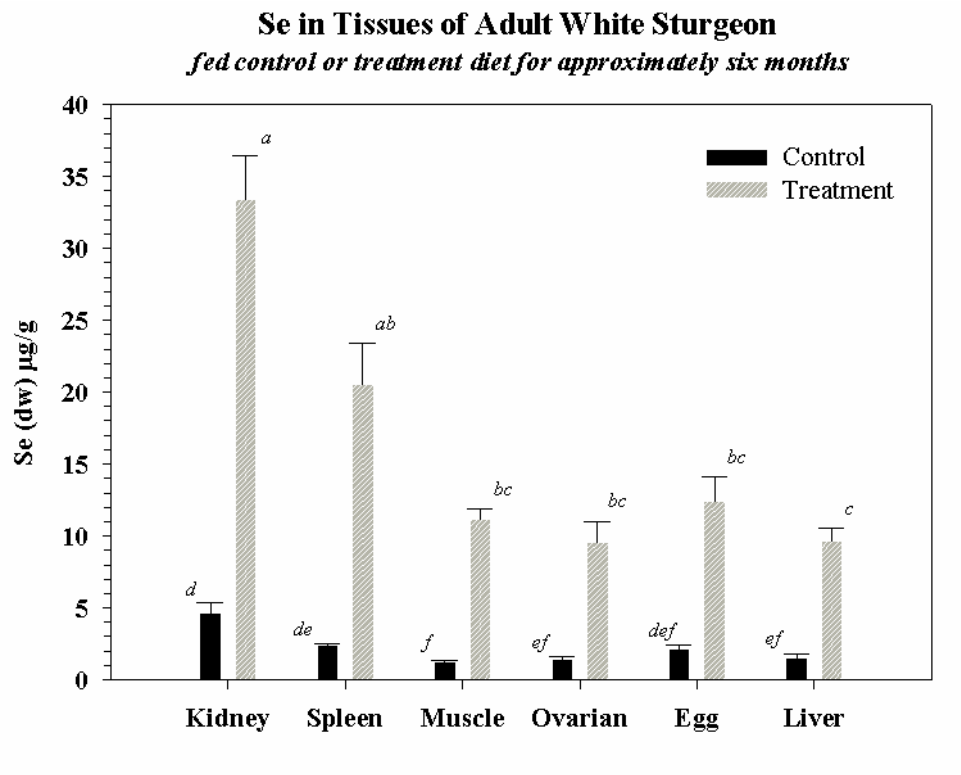


Figure 2-8. Tissue Se concentrations in white sturgeon exposed to *ca.* 1.5 or 34 µg/g dietary Se for approximately six months. Bars show mean Se µg/g, dw, with vertical lines showing standard error of the mean. Sample size is 7, except for control muscle and eggs (n = 6 and 5, respectively). Lognormal data compared by two-way nested ANOVA ($p = 0.028$) with adjusted multiple comparisons (family $\alpha = 0.05$). Groups that do not have the same letter in superscript are significantly different. Data are also presented in Table 2-4.

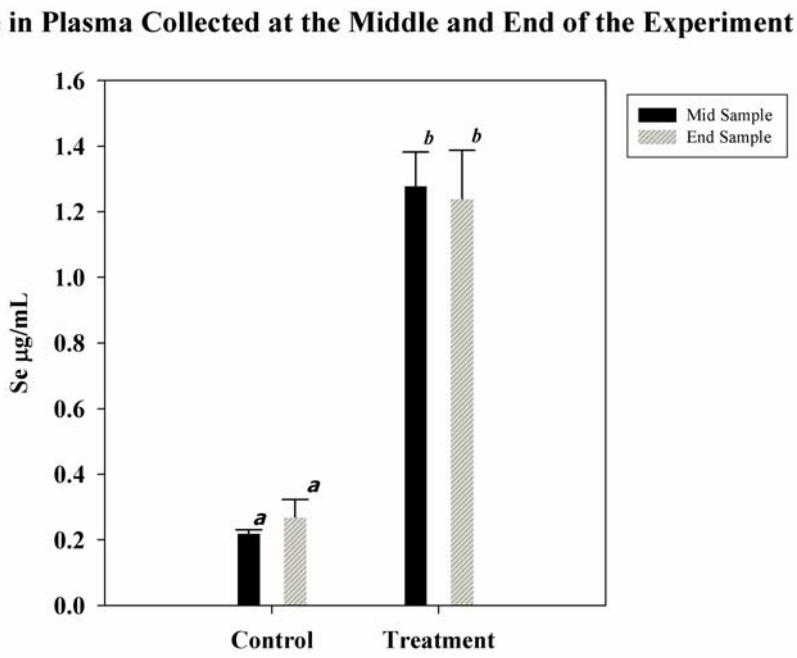


Figure 2-9. Se in plasma collected at mid- and end-experiment (after 88 and 126 - 200 days of dietary exposure, respectively) compared across exposure groups (two-way nested ANOVA, $p < 0.0001$). Bars show mean Se µg/ml with vertical lines showing standard error of the mean. Sample size is 7, except for control plasma (n = 8). Means that do not have the same letter in superscript are significantly different (Tukey HSD, $\alpha = 0.05$). Data are also presented in Table 2-6.

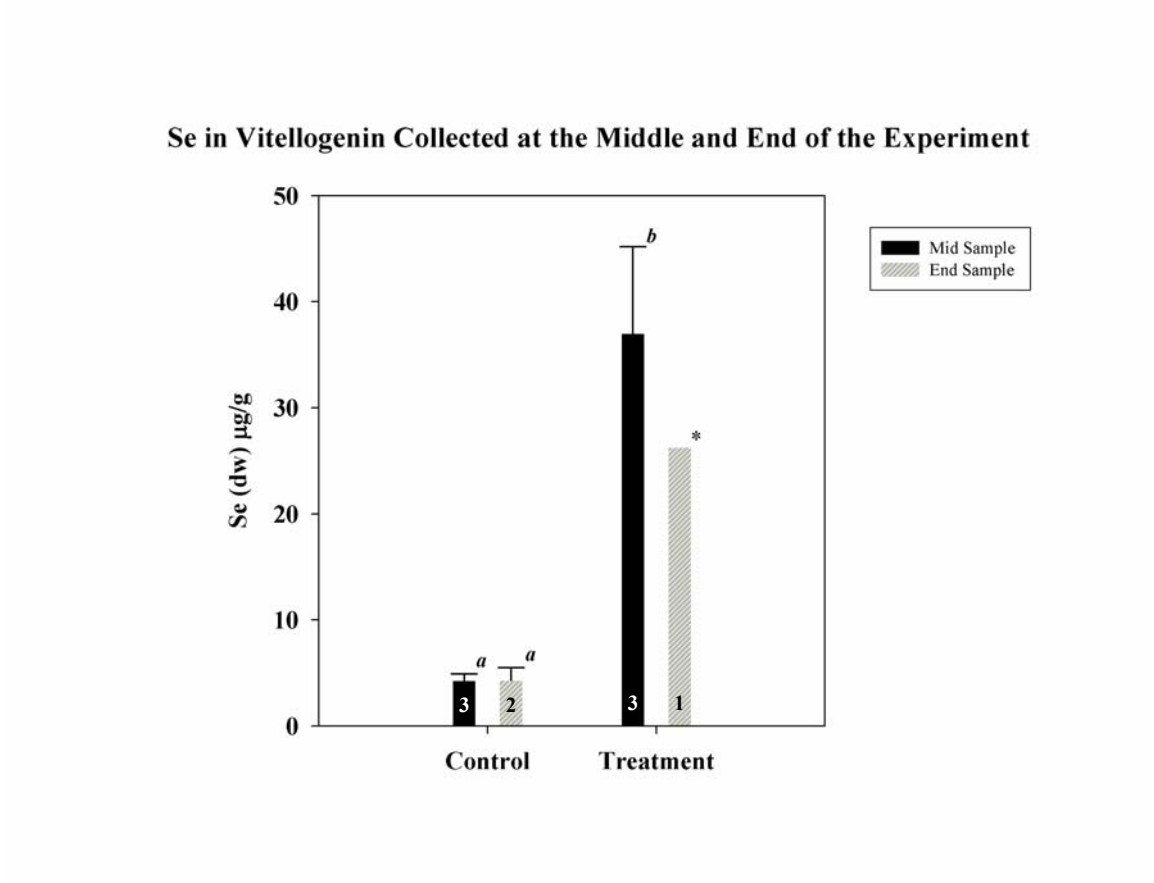


Figure 2-10. Se in VTG isolated from plasma collected at mid- and end-experiment (after 88 and 126 - 200 days of dietary exposure, respectively) compared across exposure groups (ANOVA; $p < 0.011$). Bars show mean Se µg/ml with vertical lines showing standard error of the mean. Due to small sample sizes, isolated VTG protein samples were pooled prior to Se analysis (Table 2-2). Pooled sample size shown in bars. Means that do not have the same letter in superscript are significantly different (Tukey HSD, $\alpha = 0.05$). *Only one data point was available for the end time point in the treatment group. One-sample t-tests were calculated between mean Se values and the single end data point for treatment VTG (26.29 µg/g Se). Both of the time points in the control group had mean Se values significantly different from the end-treatment data point ($p < 0.02$). The mid-treatment mean Se was not different from the end-treatment data point ($p = 0.32$). Data are also presented in Table 2-6.

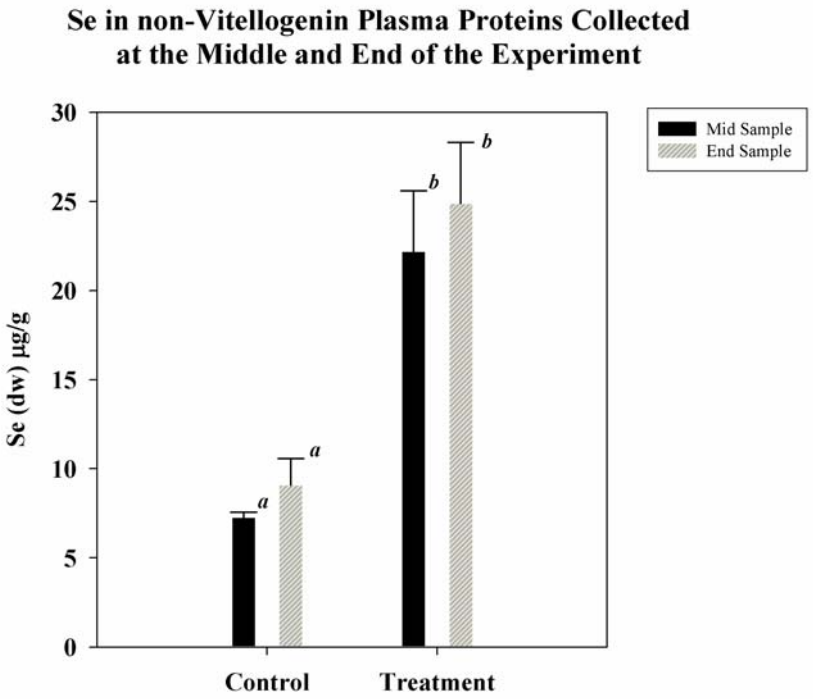


Figure 2-11. Se in nonVTG proteins isolated from plasma collected at mid- and end-experiment (after 88 and 126 - 200 days of dietary exposure, respectively) compared across exposure groups (two-way nested ANOVA; $p < 0.0001$). Bars show mean Se µg/g, dw, with vertical lines showing standard error of the mean. Sample size is 7. Data not connected by same letter are significantly different (Tukey HSD, $\alpha = 0.05$). Data are also presented in Table 2-6.

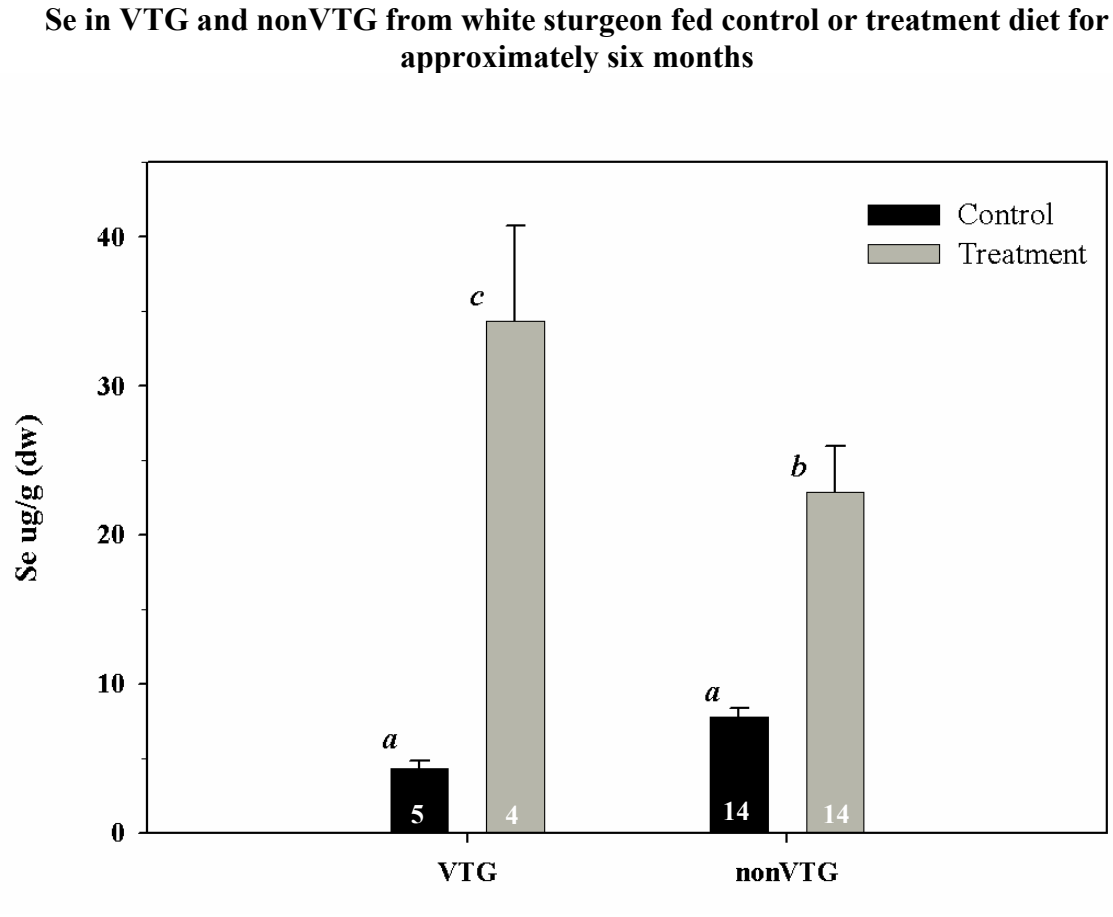


Figure 2-12. Se concentrations in VTG and nonVTG proteins. Data from both mid- and end-experiment are combined. Bars show mean Se μg/g, dw, with vertical lines showing standard error of the mean. Sample size shown in bars (VTG are pooled samples). Data not connected by same letter are significantly different (two-way ANOVA, $p = 0.032$; Tukey HSD, $\alpha = 0.05$).

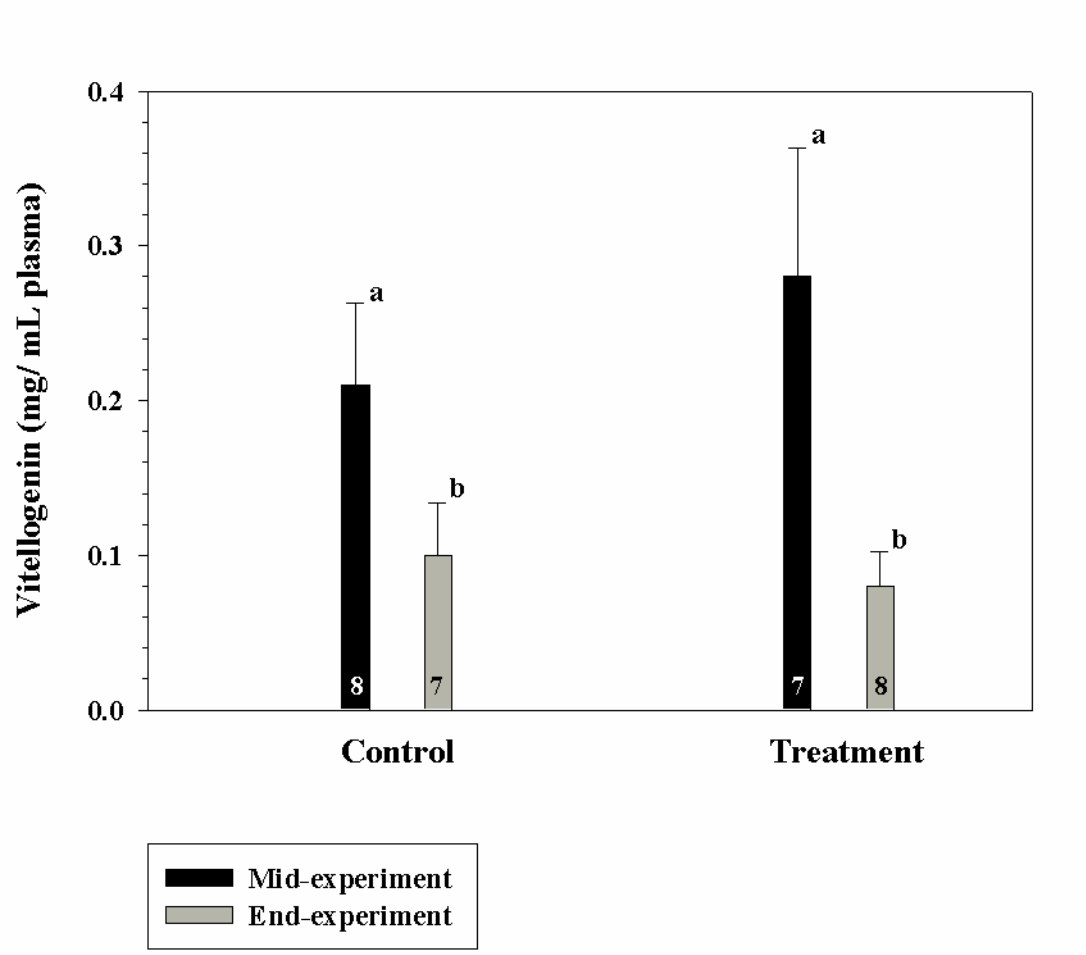


Figure 2-13. Vitellogenin isolated from plasma collected at mid- and end-experiment (after 88 and 126 - 200 days of dietary exposure, respectively). Plasma VTG concentrations were greater during mid-experiment as compared to end-experiment, but were not different between control and treatment fish (two-way nested ANOVA, $p = 0.048$; Tukey HSD, $\alpha = 0.05$). Bars show mean VTG mg/g, with vertical lines showing standard error of the mean. Sample size shown in bars. Data not connected by same letter are significantly different.

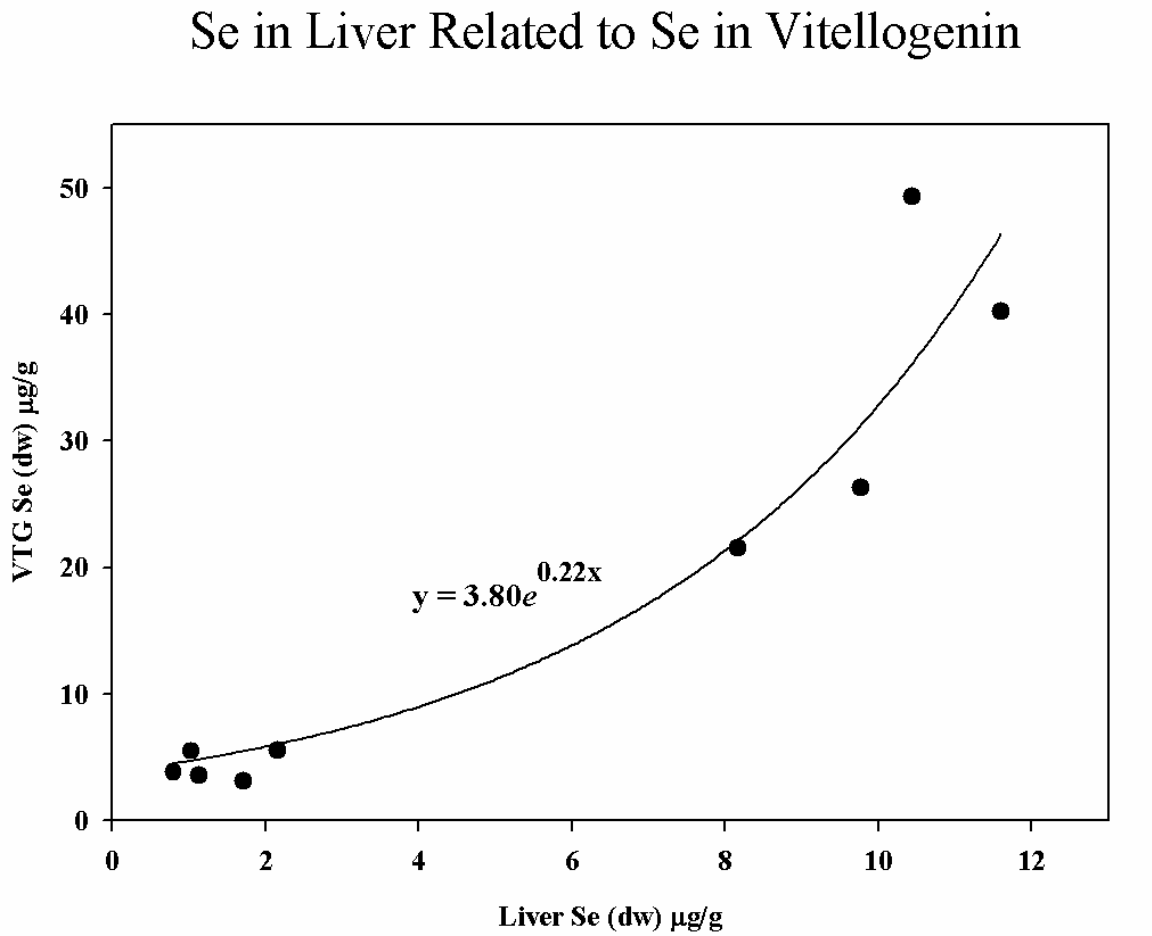


Figure 2-14. Se in VTG increases exponentially with increasing Se in liver ($R^2 = 0.90$; $p < 0.0001$). Data includes Se in VTG isolated from plasma collected at mid- and end-experiment (after 88 and 126 - 200 days of dietary exposure, respectively). All liver samples were collected at the end of the experiment. Due to small sample sizes, isolated VTG protein samples were pooled prior to Se analysis (Table 2-2). Se in pooled VTG samples is compared to the average Se in the corresponding combinations of liver data (Table 2-8).

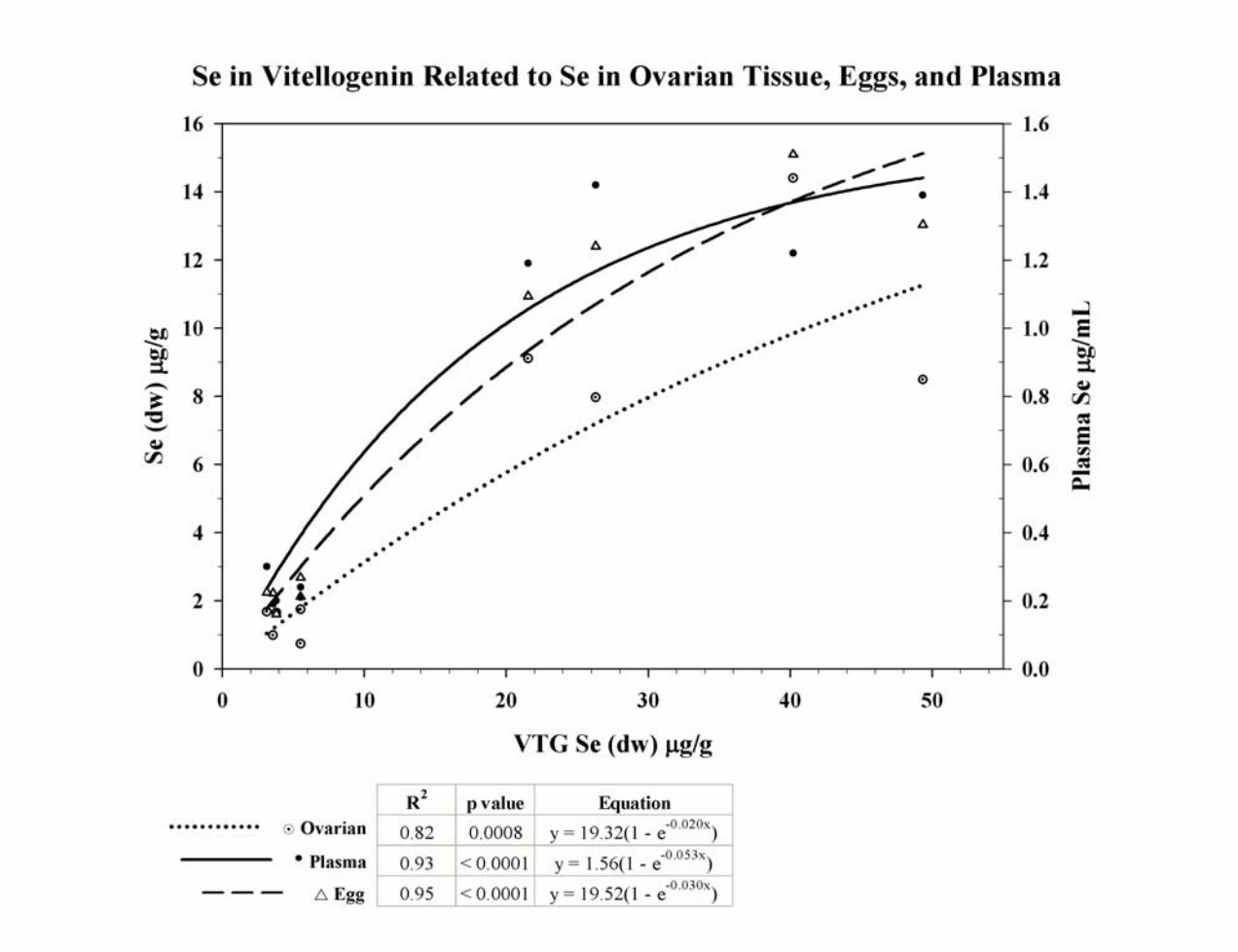


Figure 2-15. Se in plasma, ovarian tissue and eggs increases exponentially with increasing Se in VTG (weighted regressions used for ovarian and egg). Due to small sample sizes, isolated VTG protein samples were pooled prior to Se analysis (2-2). Se in pooled VTG samples is compared to the average Se in the corresponding combinations of plasma, ovarian tissue and egg data (Table 2-8). Data displayed as Se µg/g, dw, except plasma, which is presented on the right y-axis as Se µg/ml. Each regression is statistically significant.

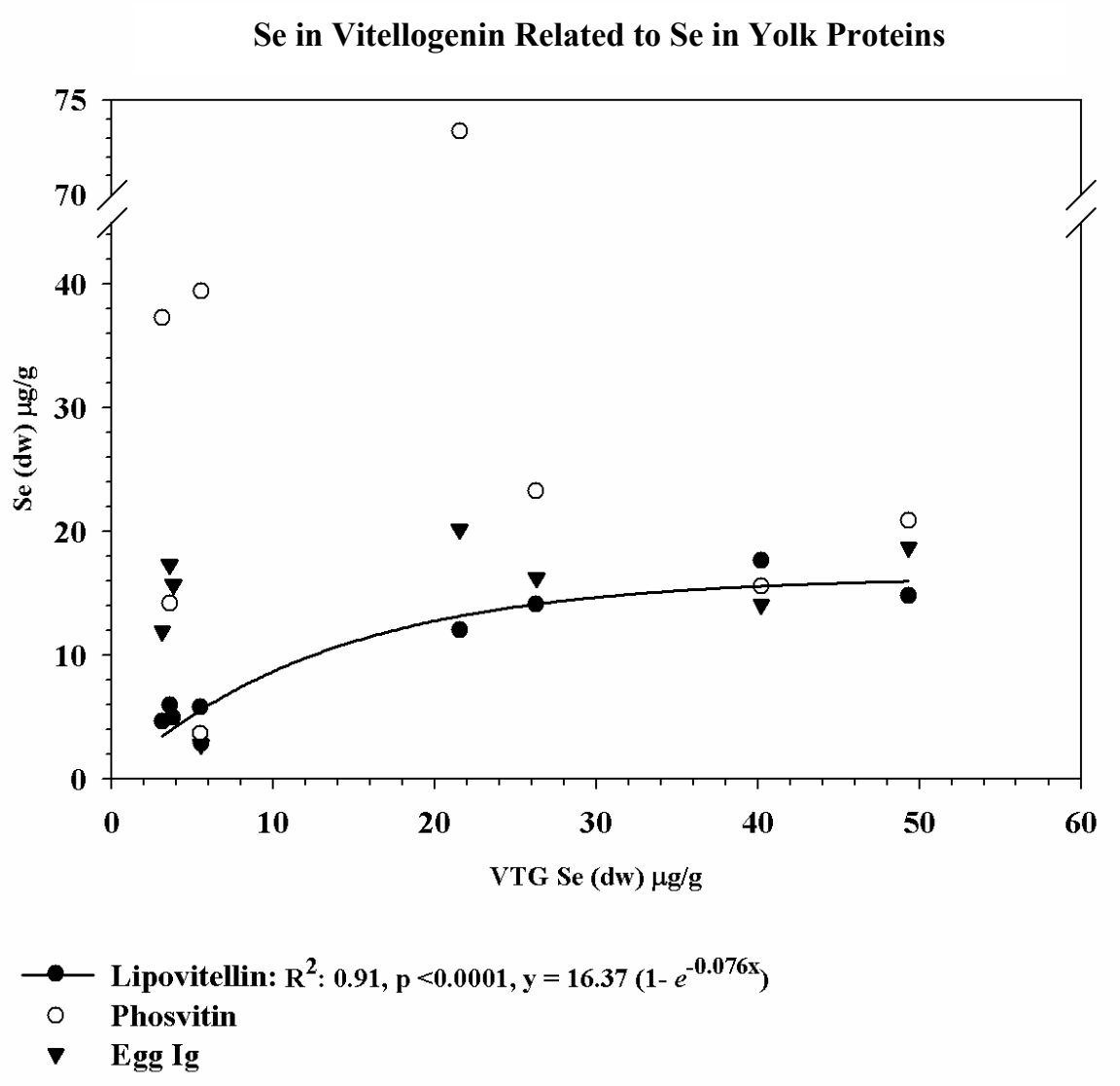


Figure 2-16. Se in lipovitellin increases exponentially with increasing Se in VTG (R^2 = 0.91; $p < 0.0001$). Se in phosvitin and egg immunoglobulin were not significantly related to Se in VTG ($p = 0.74$ and 0.32, respectively). Due to small sample sizes, isolated VTG protein samples were pooled prior to Se analysis (Table 2-2). Se in pooled VTG samples is compared to the average Se in the corresponding combinations of yolk protein data (Table 2-8). Data are displayed as Se µg/g, dw.

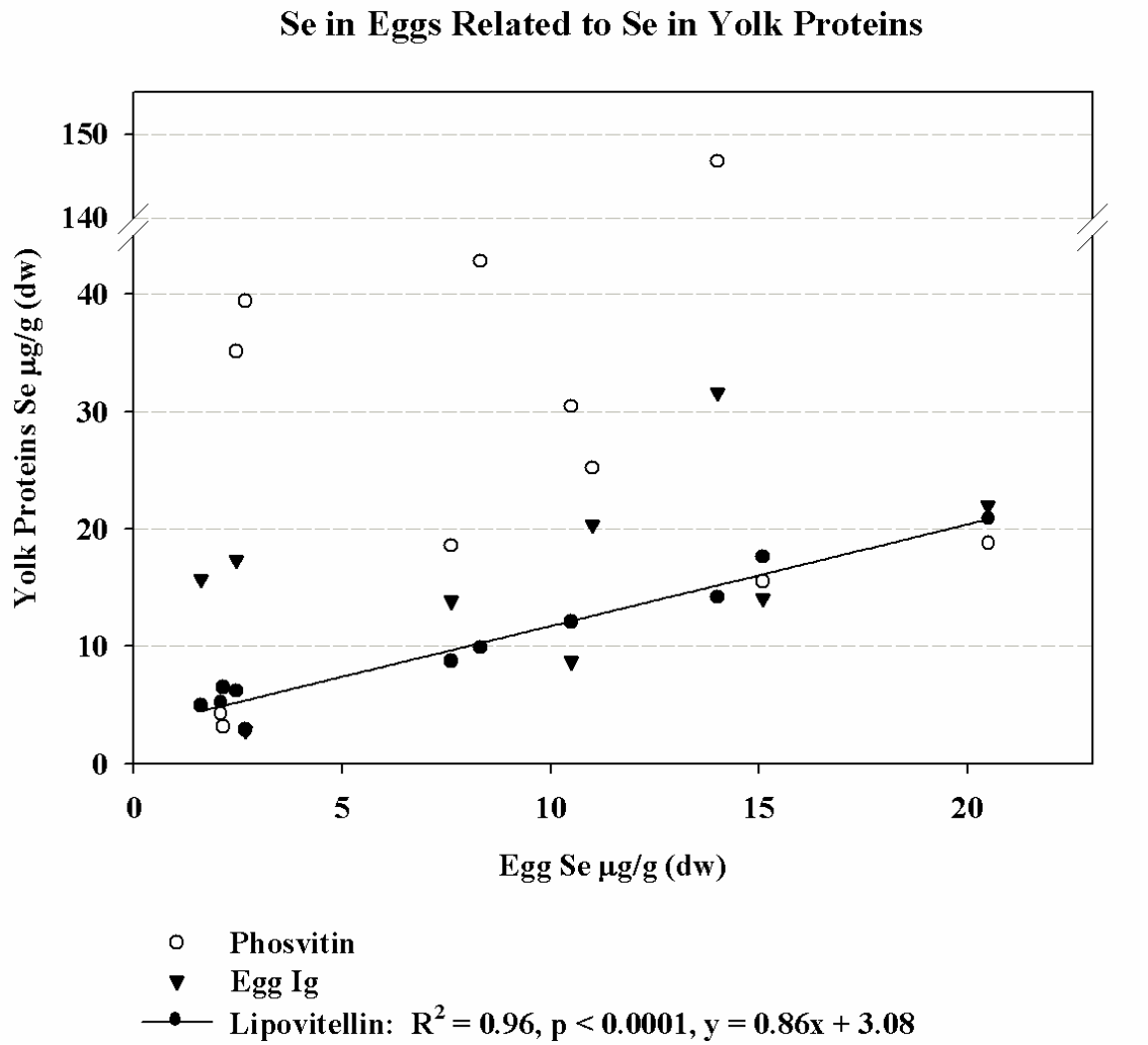


Figure 2-17. Se in isolated yolk proteins as a function of Se in eggs. A significant linear relationship was found between Se in eggs and Se in lipovitellin ($R^2 = 0.96$; $p < 0.0001$). Se in phosvitin and egg immunoglobulin were not significantly related to Se in eggs ($p = 0.14$ and 0.17, respectively).

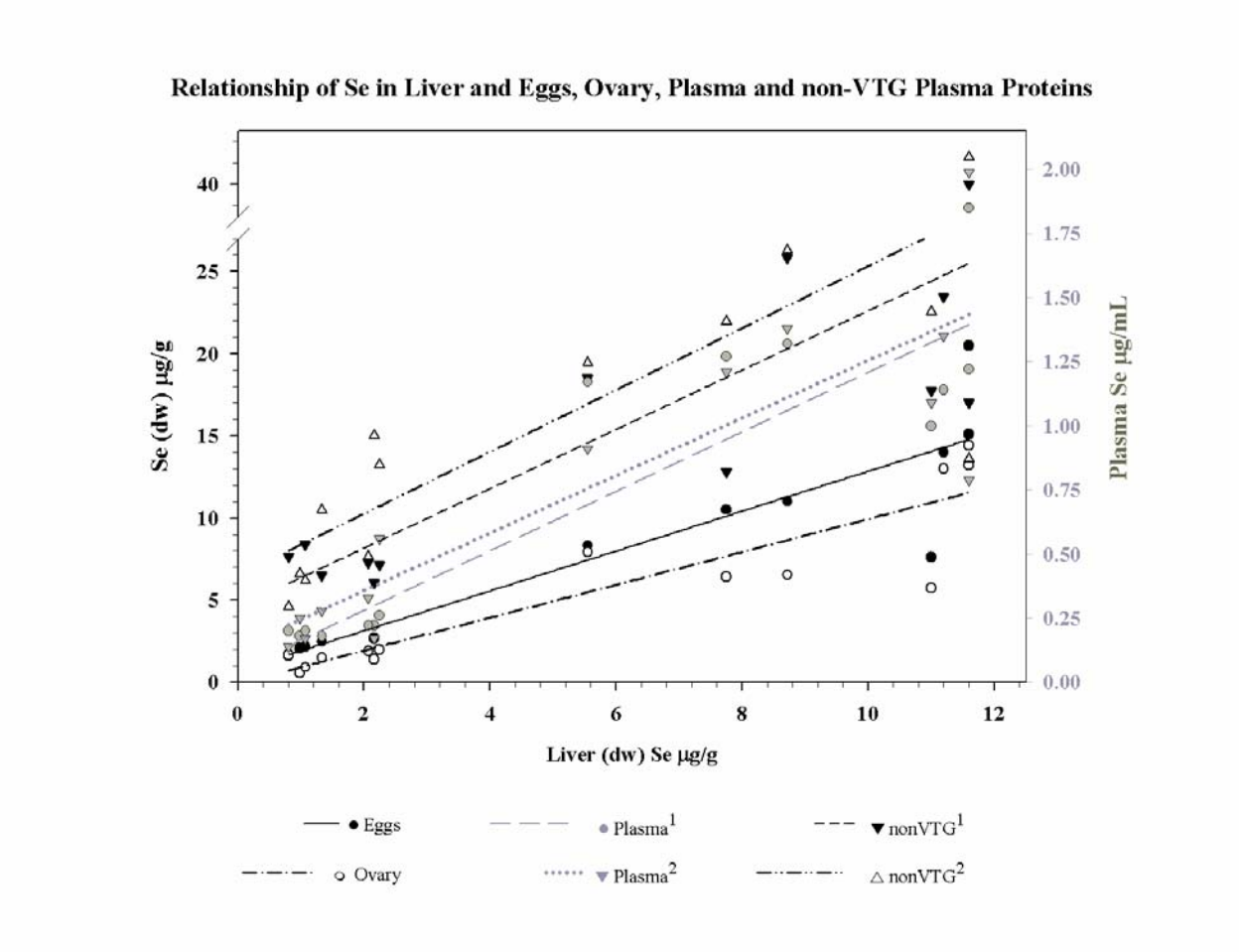


Figure 2-18. Se concentrations in ovary, eggs, plasma and nonVTG isolated plasma proteins as a function of Se concentrations in liver. [1]Sampled after 88 days of exposure. [2]Sampled at spawning or necropsy, after 126 - 200 days of exposure. Data displayed as Se µg/g, dw, except plasma, which is presented on the right y-axis as Se µg/ml. Each regression is statistically significant. Complete regression statistics are presented in Table 2-9.

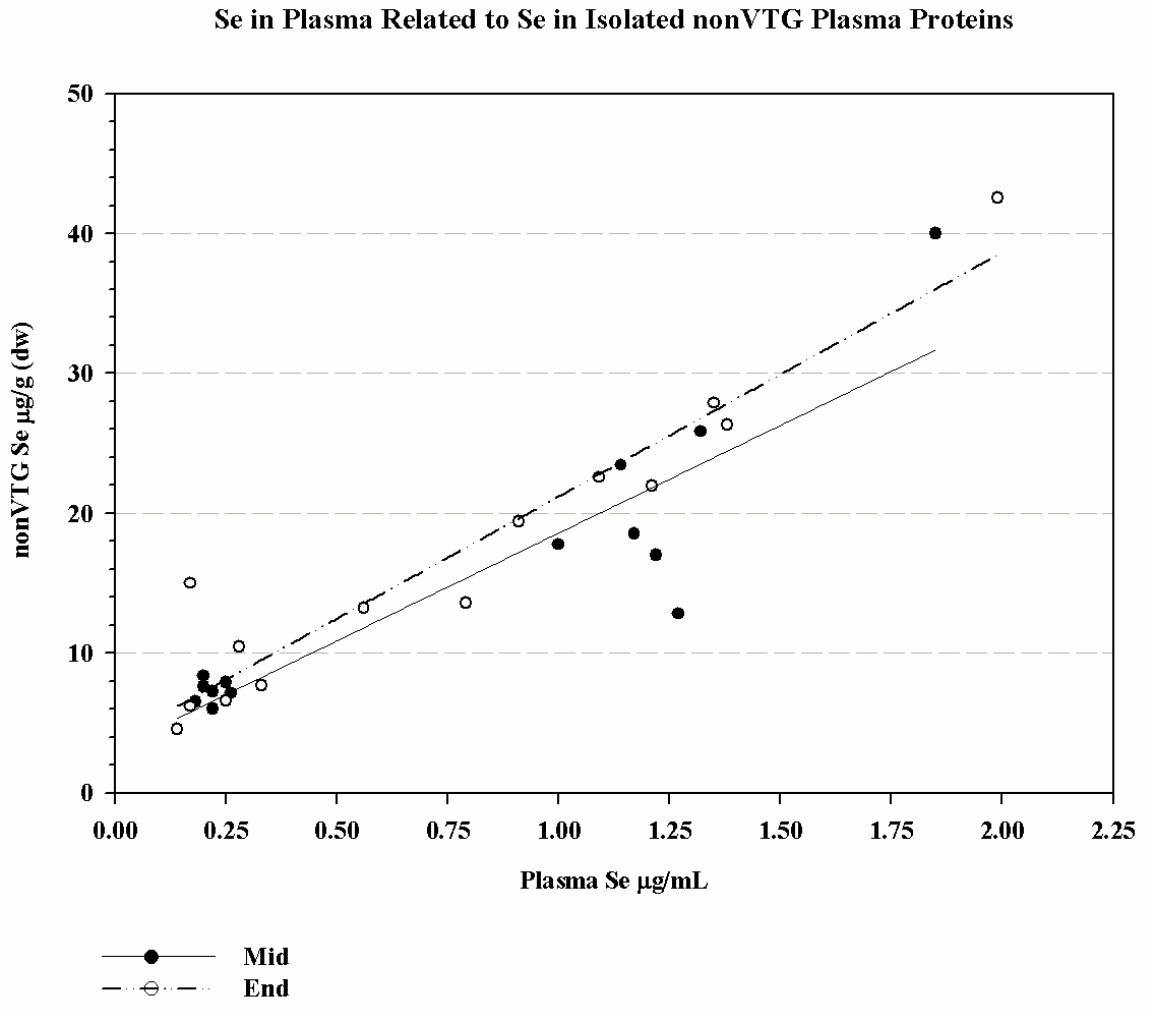


Figure 2-19. Se in nonVTG isolated plasma proteins correlated with Se in plasma. Data presented for samples collected at mid- and end-experiment (after 88 and 126 – 200 days of exposure, respectively). A statistically significant linear correlation was found between plasma and nonVTG isolated plasma proteins ($p < 0.0001$; $R^2 = 0.82$ and 0.92 for mid- and end-experiment, respectively).

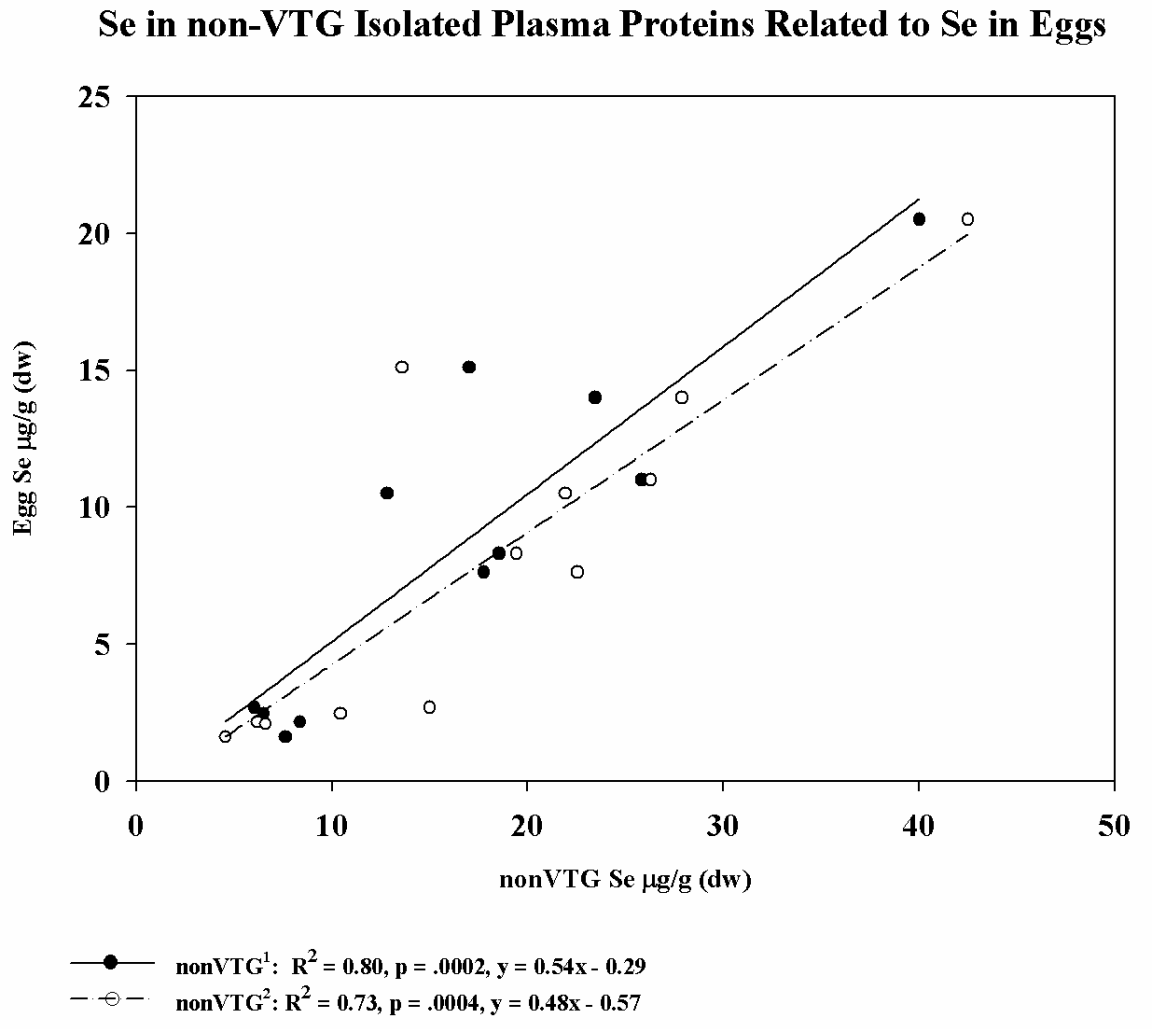


Figure 2-20. Se in eggs increase linearly with increasing Se in nonVTG isolated plasma proteins ($p < 0.0005$). NonVTG proteins were isolated from plasma collected after [1]88 days of exposure and [2]126 - 200 days of exposure. Data displayed as Se µg/g (dw).

Chapter 3. Developmental toxicity in white sturgeon larvae exposed to selenium through maternal transport or microinjection

Introduction

Selenium (Se) is a well-documented developmental toxin to fish and wildlife species (Coyle *et al.* 1993; Gillespie and Baumann 1986; Hamilton 2004; Hamilton *et al.* 1986; Hermanutz 1992; Hoffman *et al.* 1988a; Lemly 1993b; Ohlendorf *et al.* 1986). Chapter two demonstrated the maternal transfer of Se to eggs and embryos during vitellogenesis in white sturgeon. Transfer and storage of Se in the egg yolk compromises the development and survival of embryos and yolk sac larvae of several fish species (Gillespie and Baumann 1986; Lemly 1993b; Schultz and Hermanutz 1990; Woock *et al.* 1987) since they have not fully developed the organs and enzymes necessary for Se detoxification and excretion (e.g., gills, liver and kidney). If white sturgeon larvae are similarly susceptible, then recruitment may be affected in populations residing in high-Se areas.

The direct effects of Se on recruitment are difficult to detect in white sturgeon populations because monitoring is typically accomplished by tracking sub-adults and adults, which are generally at least 9 years of age (Schaffter and Kohlhorst 1999). Thus, poor recruitment is usually not detected until a decade or more after the hatching of a particular year-class. Nevertheless, Kohlhorst *et al.* (1991) have shown that recruitment success is a major determinant of white sturgeon population abundance in San Francisco Bay-Delta. White sturgeon populations in this region have been highly variable over the past five decades (Kohlhorst 1980; Kohlhorst *et al.* 1991; Schaffter and Kohlhorst 1999).

Population surveys in 2005 indicated that the San Francisco Bay-Delta white sturgeon population was at a 50-year low, with an estimated 10,000 adult sturgeon (California Department of Fish and Game Commission 2006). White sturgeon only spawn every two or more years (Doroshov *et al.* 1997), which led the California Department of Fish and Game to estimate that fewer than 2,000 of these white sturgeon were likely to spawn in 2006 (California Department of Fish and Game Commission 2006). Previous studies have hypothesized that the impact of environmental contaminants may affect recruitment success of white sturgeon in this area (Kohlhorst 1980; Schaffter and Kohlhorst 1999). The population dynamics of sturgeon make them especially vulnerable to any disruption of recruitment, for example, by Se.

San Francisco Bay-Delta receives Se through both the refining of fossil fuels and irrigation drainage from the seleniferous San Joaquin Valley. White sturgeon in this area are exposed to high levels of Se through their diet. This is evidenced by high tissue Se levels in common prey of white sturgeon (Linville *et al.* 2002; Stewart *et al.* 2004; White *et al.* 1988), as well as in sturgeon muscle, liver and eggs (Kroll and Doroshov 1991; Stewart *et al.* 2004; Urquhart and Regalado 1991; White *et al.* 1989).

The biology of white sturgeon indicates that this species would be susceptible to Se-induced developmental toxicity. The accumulation of maternal Se in the egg yolk proteins of white sturgeon was shown in chapter two of this dissertation. Sturgeon embryos hatch as underdeveloped (altricial) organisms commonly referred to as yolk sac larvae. During the first 10 days post-hatch, over eighty percent of the maternal yolk is rapidly metabolized for growth, development, and for greatly increased metabolic energy associated with constant pelagic swimming of newly emerged white sturgeon larvae

(Bolker 1993; Dettlaff *et al.* 1993; Doroshov *et al.* 1983). Vital organs such as gills, liver and kidney complete differentiation during this period. Furthermore, in contrast to the extraembryonic yolk of modern teleosts, sturgeon store and digest yolk intracellularly (Buddington and Doroshov 1986), and the nutrients are transported by a well developed embryonic vascular system comprised of the ducts of Cuvier and yolk veins (Schmalhausen 1991). In fact, the yolk is stored and digested in the endodermal tissue that ultimately differentiates into the stomach, mid-intestine, liver, and pancreas (Buddington and Doroshov 1986). The intracellular digestion and increased metabolism of yolk in sturgeon may exert profound effects on the development and survival of yolk sac larvae with elevated Se levels in maternal yolk.

The experiments described here were based on the hypothesis that white sturgeon is most sensitive to Se-induced developmental defects and mortalities during the rapid yolk utilization and organogenesis experienced in the first 10 days post-hatch (yolk sac larval phase). The experiments include both maternal exposure and microinjection exposure to Se in post-hatch larvae. In the maternal exposure experiment, embryos of females used in the Se maternal transfer experiment (described in chapter two) were reared and observed for developmental defects and mortalities. Se was shown to be incorporated into yolk proteins of these embryos in the previous chapter. In the microinjection exposure experiment, seleno-L-methionine (Se-L-Met) was injected into the yolk sac of newly hatched larvae of unexposed females. Microinjection was used because the large size and long ovarian cycle of white sturgeon make it difficult and expensive to study reproductive toxicity in this species under laboratory conditions. Microinjection mimics maternal transfer of environmental contaminants and is used as an

important tool in ecotoxicology (Black *et al.* 1985; Metcalfe *et al.* 1988). Larvae from both experiments were monitored for impacts on size, normal development and survival. Occurrence of edema, skeletal curvatures, and mortality were quantified. The maternal exposure experiment provides a causal link between Se in maternal food and developmental effects in progeny. The microinjection exposure experiment confirms threshold levels of Se developmental toxicity in white sturgeon larvae.

Materials and Methods

Maternal Exposure Study

This study used developing embryos from the maternal Se exposure study described in chapter two of this dissertation. Briefly, sixteen domestic broodstock white sturgeon females (*ca.* 22.71 kg and 134.59 cm) were exposed to either control (*ca.* 1.4 µg/g Se) or treatment (*ca.* 34 µg/g Se) diets for approximately 6 months (Table 3-1; all Se values reported here are based on dry weight, unless otherwise noted). Upon acquisition of maturation competence, the fish were induced to spawn and ovulated eggs were fertilized with sperm from non-treated males. Fertilized eggs were placed into individual MacDonald hatching jars; and jars were set up to allow hatched larvae from individual females to flow into separate 1.2 m diameter circular tanks. Embryo incubation was at water temperatures of 15 to 16 ºC.

After hatching, larvae were acclimated from 16 ºC to 18 ºC over one day. Larvae were then moved to an indoor shelter at the Center for Aquatic Biology and Aquaculture (CABA) at the University of California, Davis (UCD) and placed into flow-through buckets secured in 1.2 m diameter circular flow-through tanks. Flow-through buckets

were fashioned from 19 liter high-density polyethylene buckets (*ca.* 26 cm diameter, 37 cm depth) with screens covering large windows (*ca.* 25 by 30 cm) on each side to provide a constant cross-flow.

For each progeny cohort, 3,000 larvae were randomly distributed into flow-through buckets, three replicate buckets for stage 40 sampling (n=500) and three replicate buckets for stage 45 sampling (n=500). A pair of stage 40 and 45 replicate flow-through buckets was placed in three separate tanks to control for any tank effects. Separate tanks were used for control and treatment group larvae. Temperature was maintained at 18.56 ± 0.13 °C. Flow rate in tanks ranged from 1.0 to 1.5 L/min. Dissolved oxygen ranged between 90 to 95% saturation in all tanks. Natural photoperiod was maintained by artificial light, and approximately 80% of light was blocked by shade-cloth tank covers. Larvae were observed daily for mortality, morbidity and developmental abnormalities.

Sampling

Key sturgeon developmental stages were used to time sample collections for tissue Se and to conduct morphological analysis of larval development (stages 36 – 45, yolk sac larvae). Table 3-2 shows developmental staging following Dettlaff *et al*. (1993) for Russian sturgeon, with specific characteristics for white sturgeon described by Beer (1981) and Deng (2000). There were slight variations in stage within samples due to the fast rate of development. Samples were taken for Se analysis at stages 5, 21, 33 (embryos) and 36, 40, and 45 (larvae). Developmental defects and size were assessed at stages 36, 40 and 45. Sampled larvae were anesthetized in 150 ppm MS-222, overdosed in 500 ppm MS-222, rinsed in millipure water, then either frozen on dry ice and stored at

–80 °C (for Se analysis), fixed in 10% buffered formalin (for developmental assessment), or measured immediately (for size measurement). All post-hatch developing white sturgeon are referred to as larvae in this chapter. The term larval development is used here to describe the rapid development that takes place between hatching and the initiation of exogenous feeding (stages 36 – 45).

Selenium analysis

Approximately 60 – 90 individual embryos or larvae were homogenized in millipure water using a hand blender and then freeze-dried. Approximately 300 mg of tissue was digested in a mixture of concentrated sulfuric, nitric and perchloric acids with a gradual temperature increase to 330 ^{o}C. The samples were then reduced by concentrated hydrochloric acid heated to 95 ^{o}C and measured as selenite by hydrogen generation inductively coupled plasma-atomic emission spectrometry (ICP-AES). The minimum detection limit was typically 5 ng/ml. Quality control measures included the use of standard reference materials (NRC DORM-2, DOLT-2, DOLT-3), spiked samples, duplication and blanks with every analytical run. All quality control measurements agreed with the target concentration within 20 percent.

Assessment of larval development

Preserved larvae were rinsed with millipure water and visually observed for gross developmental abnormalities. The development was monitored using the morphological criteria described by Dettlaff *et al.* (1993) for Russian sturgeon, with specific characteristics for white sturgeon described by Beer (1981) and Deng (2000; Table 3-2).

The occurrence of edema (overt swelling caused by accumulation of fluid), lordosis (concave dorsoventral curvature of posterior region), kyphosis (dorsoventral curvature of anterior region), and scoliosis (lateral skeletal curvature) was recorded for each sample. Stage 36 samples contained approximately 30 individuals. Samples were collected from each of the three stage 40 and 45 replicate buckets (six buckets per female); each replicate sample contained approximately 30 individuals.

Measurement of larval size

Body weight and total length were measured in approximately 30 larvae of each progeny cohort for three time points. Deformed larvae were not excluded from these measurements. Total length was measured under a dissecting scope, using a digital image tablet (± 0.01 mm). Larvae were blotted and weighed individually (wet weight) on a microbalance (± 0.0001 g) then placed in tared whirl packs, lyophilized, and weighed to determined dry weights.

Microinjection Studies

Study One

Post-neurulated embryos were obtained from spawning of domestic stock (Columbia River origin) at Clear Springs Food Co. (Idaho). Eggs were fertilized on May 19, 2004, and post-neurulated embryos shipped to California in sealed plastic bags with hatchery water (14.5 °C) and oxygen. Upon arrival, the embryos (stage 30-31; Dettlaff *et al.* 1993) were acclimated to 15.7 °C for two hours and transferred to crystallizing dishes

submerged in tanks. Approximately 90% of post-neurulated embryos received from Idaho survived to hatching and provided viable yolk sac larvae for this experiment.

Newly hatched larvae (stage 36) with normal morphology and behavior (Beer 1981; Dettlaff *et al.* 1993) were used for microinjections. Microinjections took place on May 28, 2004 following the methods described in detail below. The control larvae underwent the same procedures (anesthesia and microinjection with sterile millipure water). In addition, a second control group of anesthetized but non-injected larvae was used, to account for the physical effect of microinjection. Following injection, larvae were allowed to recover in fresh water at 15.7 °C before stocking in tanks. The maintenance of the larval culture is described in detail below.

Se-L-Met was dissolved in autoclaved vials using sterile millipure water to 7.0, 14.1, and 28.2 mg/mL. Target doses were calculated as described below. The injection volume was 25 nL per larva, resulting in Se dosing of 10, 20 and 30 μg/g (dw). Controls consisted of larvae injected with 25 nL of sterile millipure water (sham-injected) and non-injected larvae. A total of 160 larvae were injected in each treatment and control groups. Within each group the 160 larvae were randomly distributed into four tanks; three replicate survival tanks (n=35) and one sampling tank (n=55). All replication was assigned to tanks by using a random number table.

Animals in survival tanks were monitored daily for mortality and morbidity. Sampling tanks were monitored daily for physical (e.g., heartbeat, pericardial edema, yolk absorption) and morphological (e.g., skeletal) abnormalities of larvae. Larvae from each treatment were collected from each sampling tank one day post injection and at the endpoint of the experiment (stage 45, yolk depletion) for Se analysis and morphology.

Sampled larvae were anesthetized in 150 ppm MS222, overdosed in 500 ppm MS-222, rinsed in millipure water, then frozen on dry ice and stored at –80 °C (Se analysis) or fixed in 10% buffered formalin containing 4% sucrose (morphology).

Study Two

White sturgeon ova of two females and milt (pooled from 4 males) were obtained from a commercial farm (Stolt Sea Farms, CA). The samples of ova (approximately 50 ml) and pooled milt (20 ml) were transported 1 hour to UCD in a temperature-controlled chamber at 15 °C. Upon arrival, sperm were examined for motility.

Eggs of two females (referred to here as Female 1 and Female 3) were fertilized (1:100 milt dilution), silted with 5 ppt Fuller's earth (Sigma) for 1 hour and incubated in tanks in a flow-through water (12.7 °C) re-circulating system. At start of hatching, temperature was gradually increased to 15.2 °C. Microinjections were initiated after 90% of the larvae had hatched. Microinjection of newly hatched larvae (stage 36) was performed on June 21, 2004 as described below. Non-injected control larvae were subjected to anesthesia and similar handling procedures. After injections, larvae were allowed to recover in fresh water at 15.7 °C before stocking in rearing tanks. The maintenance of the larval culture is described in detail below.

Se-L-Met was dissolved in sterile vials using sterile millipure water to 2.11, 6.34, and 19.03 mg/mL. Target doses were calculated as described below. The injection volume was 25 nL per larvae, so the target Se doses were 3, 9 and 27 µg/g (dw). Controls were non-injected, sham-injected (25 µL millipure water), and L-Met injected larvae (as a control for increased exposure to methionine). Three concentrations of L-

Met (MW 149.2; Sigma-Aldrich, St. Louis, MO) were used for additional control (2.11, 6.34, and 19.03 mg/g).

For each progeny, a total of 445 larvae were used (365 injected and 80 non-injected). Non-injected, sham-injected, and low, medium, and high Se-L-Met groups of 80 larvae were each distributed into one survival (n=30) and one sampling (n=50) tank. For low, medium, and high L-Met groups, 30 larvae of each group were stocked in a survival tank. All tank assignments were random.

Daily monitoring of survival and abnormalities was similar to *study one*. Larvae were randomly sampled for morphology and Se content at two hours post injection (stage 36), at stage 40 (pyloric sphincter formation), and at stage 45 (yolk depletion). Sampled larvae were anesthetized in 150 ppm MS222, overdosed in 500 ppm MS-222, rinsed in millipure water, then frozen on dry ice and stored at –80 °C (for Se analysis) or fixed in 10% buffered formalin containing 4% sucrose (for morphology).

In addition, each treatment was sampled at the start and endpoint of the experiment to determine live body weight and total length. Total lengths of newly hatched larvae were measured in 10-15 individuals under a dissecting scope, using a digital image tablet (± 0.01 mm). Larvae at the yolk depletion stage (stage 45) were measured with a caliper (± 0.5 mm). Larvae were blotted and weighed individually (wet weight) on a microbalance (± 0.0001 g) then placed in tared whirl packs, lyophilized, and weighed to determined dry weights.

Larval culture maintenance

Microinjection experiments were carried out at CABA at UCD. Indoor flow-through water recirculation systems with biological filter, aeration, YSI thermostat, chiller, and heater were used for all phases of the experiments. Eggs and larvae were held in circular fiberglass tanks (28 cm diameter, 35 cm depth, flow rate 1.0 – 1.5 L/min). Dissolved oxygen and temperature were recorded daily. A certified calibrated thermometer (National Institute of Standards and Technology) was used to adjust incubation temperature to 15.7 ± 0.1 °C. Dissolved oxygen was above 90% saturation in all tanks. Natural photoperiod was maintained by artificial light, and approximately 80% of light was blocked by shade-cloth tank covers. Ammonia in water ranged from less than 0.003 to 0.014 mg/L NH_3 and pH ranged from 8.2 to 8.3 (DNRA laboratory, UCD). Se concentration in the water of recirculation systems was below 1.3 µg/L at the initiation of experiments and 1.2 µg/L at the endpoint of experiments (Flame AA on digested samples, SM3114B and SM303E; Sierra Foothill laboratory, Inc., Jackson, California; certified for water Se analysis at UCD facilities).

Key sturgeon developmental stages were used to initiate treatments, to collect tissue samples for Se and to conduct morphological analysis at midpoints and endpoints of each experiment. Table 3-2 shows developmental staging following Dettlaff *et al*. (1993) for Russian sturgeon, with specific characteristics for white sturgeon described by Beer (1981) and Deng (2000; Table 3-2). There were slight variations in stage within the sample due to the fast rate of development.

Microinjection

Newly hatched larvae (stage 36) with normal morphology and behavior (Beer 1981; Dettlaff *et al.* 1993) were anesthetized in 150 ppm solution of MS-222 at 15.7 °C for approximately one minute. The 100 x 15 Integrid Petri plate (Falcon #1012) was turned upside down and a water saturated Whatman filter paper (90 x 90 cm) placed on top. Using a transfer plastic pipette, ten larvae were lined up in two columns on the paper top for microinjection. The injection site was the upper mid portion of the yolk sac, between the duct of Cuvier and yolk veins, to avoid puncturing the major blood vessels. On average 10 larvae were injected within 2 minutes. After injections, larvae were allowed to recover in fresh water at 15.7 °C before stocking in tanks.

Microinjections were performed using a pressure injector (programmable picoinjector IM 300, Narishige Group, Japan), a joystick micromanipulator (MN-151, Narishige group, Japan) mounted on a magnetic stand, and aluminosilicate needles (A150-100-10, Sutter Instruments Co., Novato, CA). Needles were pulled from aluminosilicate tubing (1 mm OD, and 0.67 mm ID) using a programmable Flaming/Brown micropipette puller (P-97, Sutter Instruments Co., Novato, CA) to obtain a 10 μm OD and 100 to 150 μm long needle tips. Pulled needles were coated (Sigmacote, Sigma, St. Louis, MO), beveled (EG-44 Microgrinder, Narishige Group, Japan), back filled with approximately 5 μL of injection solution followed by an oil plug of *ca.* 10 μL (paraffin oil, #76235, Fluka Production, Germany) using Eppendorf microloaders. Each needle was individually calibrated using a stereoscope (Wild M38) at 400 magnification, equipped with a pre-calibrated eyepiece micrometer. Needle calibration was done by injecting solution into paraffin oil and measuring the dispensed

drop with the eyepiece micrometer. Volume adjustment was done by increasing or decreasing injection time while maintaining the pressure constant at about 20 psi. The target nanoinjection volume throughout the experiments was 25 ± 2.5 nL (approximately 0.1 % volume of the average size white sturgeon egg). All injections were done under a stereoscope at 160 total magnification (Wild M38), with an X-Y movable stage equipped with a plate holder.

Selenium dosing

Se-L-Met (> 98% pure, product # S3132, Sigma-Aldrich, St Louis, MO) was used for the microinjection experiments. Amount of Se-L-Met (MW 196.1) used for the different treatments was calculated based on the target Se dosing per dry weight basis. The newly hatched larvae (stage 36) is 7.35 ± 0.06 and 7.03 ± 0.09 mg (dw) when incubated at 14 and 17 °C respectively (Wang *et al.* 1985). For calculations of approximate doses of Se, and preparation of stock solution, we assumed the average white sturgeon hatched larvae to be 7.1 mg (dw) for our temperature 15.7 °C.

Selenium analysis

Larvae were frozen on dry ice and stored at -80^{o}C until preparation for analysis. Each sample (n = 10) was freeze-dried and homogenized using a 0.5 ml mortar and pestle. The homogenates were repeatedly freeze-dried and approximately 2 mg of dry material was used for analysis. Total Se concentrations were analyzed by micro-digestion followed by fluorometry (Fan *et al.* 1998). Samples were digested in a mixture of nitric and sulfuric acids and gradually heated to 130 ^{o}C. The samples were then

reduced to selenite in 6 N HCl heated to 110 °C. The selenite was derivatized to piazselenol by the addition of 0.1% diaminonaphthalene (DAN) reagent (prepared by sonicating 2,3-diaminonaphthalene hydrochloride, Dojindo Laboratories (Japan), in 0.1 N HCl for 1 h, filtered through 0.45 μm cellulosic filter, and washed three times with excess cyclohexane) and extracted using 0.5 ml of cyclohexane. This mixture was incubated at 45 °C in the dark for 30 min, and then shaken to complete the extraction into the cyclohexane layer. Two hundred microliters of the cyclohexane layer was measured for the fluorescence intensity of the piazselenol derivative using a spectrofluorometer. The minimum detection limit was typically 1 μg/L Se. Quality control measures included the use of spiked samples, duplication and blanks with every analytical run. Quality control measurements agreed with the target concentration within 15 percent. Post-injected larvae of microinjection *study two* were analyzed by hydride generation ICP-AES, following the methods described above for the maternal experiment.

Assessment of larval development

Larvae were assessed visually for gross developmental abnormalities throughout the experiment and in preserved larval samples. The development was monitored using the morphological criteria described by Dettlaff *et al.* (1993) for Russian sturgeon, with specific characteristics for white sturgeon described by Beer (1981) and Deng (2000). The occurrence of edema, lordosis, kyphosis, and scoliosis was recorded. Developmental defects observed in mortalities were included in total occurrence rate.

Statistical Analysis

Data analysis was performed using JMP-In v. 5.1 (SAS Institute Inc.). Data transformations were used when necessary to meet the assumptions of parametric models. Nonparametric tests were used when the assumptions of parametric models could not be met. Measurements of larval size were compared between exposure groups using one-way nested ANOVA (with data transformation), Kruskal-Wallis, or Rank F-Test (Neter *et al.* 1996). All hypothesis multiple comparison testing was performed with either the Tukey-Kramer honestly significant difference (HSD), Dunnets, or least squares means Student's t-test, at a significance level of $\alpha = 0.05$. A two-way nested ANOVA model was applied to Se concentrations in developing white sturgeon following log-transformation for data from the maternal experiment and no transformation for microinjection data. All Se values are based on dry weight, unless otherwise noted. Developmental defects were compared using logit analyses. The logit equations were used to estimate median effective doses (ED_{50}) for developmental defects. ANOVA models were also applied to percent developmental defects (two-way nested ANOVA for maternal data, one-way ANOVA for microinjection data). This proportional data was first angular transformed as $\theta = \arcsin \sqrt{(Y + {}^{3}/_{8} / n + {}^{3}/_{4})}$, where Y is the observed number of affected individuals and n is the sample size, following Anscombe (1948). This transformation accommodates data with several near-zero values (Zar 1999). Wilcoxon Rank Sums or Rank F-Tests were used for nonparametric data. For the hypothesis comparisons of developmental effects in microinjection *study two*, the control non-injected and sham-injected groups were not significantly different and were combined into one group. Similarly, the high, medium and low L-methionine samples

were combined into one group since they were not significantly different from each other. Hypothesis comparisons of developmental effects were not possible in microinjection *study one* because only one progeny replicate was used. Kaplan-Meier survival analysis was applied to mortality data. The mean and standard deviation of Kaplan-Meier distributions were compared using the least squares means Student's t-test.

Results

Size of Developing White Sturgeon

Maternal Exposure

No differences were found in weight or length of larvae from female white sturgeon exposed to dietary Se between exposure groups at any of the larval developmental stages (ANOVA or Rank F-Test, $p > 0.2$; Table 3-3).

Microinjection

The weights and lengths of larvae from microinjection *study one* were not measured. At the start of microinjection *study two*, newly hatched Female 1 larvae had significantly greater weight (Wilcoxon Rank Sums; $p < 0.0001$) but were shorter (ANOVA; $p = 0.006$) than Female 3 larvae (Table 3-4a). Size comparisons at the end of microinjection *study two* indicate that larvae from the high Se-L-Met treatment (*ca.* 16 μg/g Se) weigh less than all other groups (nested ANOVA, $p < 0.0001$; Tukey HSD, $\alpha = 0.05$; Table 3-4b). However, the high Se-L-Met sample only included larvae from Female 3, which had lower initial weight than larvae from Female 1. This was due to the

limited sample size of this experiment and the prioritization of other analyses. Therefore, the actual affect of high Se-L-Met on larval weight is unclear.

Selenium in Developing White Sturgeon

Maternal Exposure

Se concentrations in developing white sturgeon were significantly greater in the treatment group for each developmental stage sampled (two-way nested ANOVA, $p < 0.0001$; Tukey HSD, $\alpha = 0.05$; Table 3-5; Figure 3-1). Comparisons within each exposure level showed no difference in Se concentrations between eggs, embryos and larvae (two-way nested ANOVA, $p < 0.0001$; Tukey HSD, $\alpha = 0.05$; Table 3-5). All Se values are based on dry weight, unless otherwise noted.

Microinjection

Table 3-6 and Figure 3-2 show Se concentrations in larvae of both microinjection experiments. In microinjection *study one* Se concentrations are shown for larvae one day after microinjection (*ca.* stage 36) and at the experimental endpoint (stage 45). Control groups (endogenous Se) exhibited 2.60 (non-injected) and 2.54 (sham-injected) Se (µg/g) at the initiation of the experiment. Post-injection Se concentrations in low, med and high treatment groups were 15.8, 21.7 and 46.6 µg/g respectively, approximating target Se doses of 10, 20 and 40 µg/g. At the end of the experiment, Se was measured in the survivors of each group. High (8.99 ± 0.34 µg/g) and medium (10.70 ± 0.34 µg/g) Se treatment groups had lower concentration of Se than at the start of the experiment (Se shown as mean ± standard error of three replicate tanks). The low treatment group larvae

contained 13.92 ± 1.03 µg/g Se at the experimental endpoint. Control non-injected and sham-injected groups exhibited 5.43 ± 0.14 and 5.19 ± 0.17 µg/g Se, twice as much as the start of the experiment. The discrepancy may relate to two different methods of Se analysis and different sample sizes at the start and end of the experiment (micro-digestion with Fluorometry and n of 5 – 10 at start; macro-digestion with ICP-AES and n of *ca.* 45 at end; Table 3-6). There was insufficient data (one progeny replicate) to perform a statistical comparison of the above Se concentrations.

In microinjection *study two* Se concentrations are shown for larvae 1 to 2 hours post injection (*ca.* stage 36), mid-experiment (stage 40), and end-experiment (stage 45; Table 3-6). Control groups (endogenous Se) exhibited 6.35 ± 0.35 (non-injected) and 5.89 ± 0.77 (sham-injected) Se (mean µg/g ± standard error of the two progeny cohorts) at the start of the experiment. Post-injection Se concentrations in low, med and high treatment groups were 8.74 ± 0.36, 8.96 ± 0.2, and 16.55 ± 1.62 µg/g respectively, which differed from target exposure levels of 3, 9 and 27 µg/g Se. At 1 to 2 hours post injection and at mid-experiment (stage 40), larvae in the high treatment group contained significantly higher Se than the other treatment groups and controls (two-way, nested ANOVA, $p < 0.0001$; Tukey HSD, $\alpha = 0.05$; Figure 3-2). Additionally, a separate analysis of the mid-experiment data showed that Se concentrations in each of the three treatment groups were significantly higher than in the controls (ANOVA $p < 0.002$; Dunnets $\alpha = 0.05$; Figure 3-2). No other significant differences were observed between Se concentrations between experimental groups or time points.

Developmental Defects in Larvae

Maternal Exposure

Developmental defects measured included edema and the skeletal deformities, lordosis, kyphosis, and scoliosis (Figure 3-3). Approximately 1,000 larvae from a total of six females were measured for developmental defects. Logit analysis showed significant associations between the occurrence of edema ($\chi^2 = 39.7$) and skeletal deformities ($\chi^2 = 46.3$) and Se concentrations in larvae ($p < 0.0001$; Tables 3-7 & 3-8; Figures 3-4 & 3-5). Logit probabilities were used to estimate that 10, 25 and 50 percent of the population would incur edema at *ca.* 21, 25, and 30 μg/g Se in larvae, respectively (Figure 3-4). Skeletal deformities would be expected in 10, 25 and 50 percent of the population at *ca.* 17, 24, and 32 μg/g Se in larvae, respectively (Figure 3-5). Either of the effects, edema or skeletal deformities, would significantly reduce the chances of survival in the wild. Thus, logit analysis was also applied to the occurrence of edema and/or skeletal deformities in order to measure the total number of affected larvae. There was a significant association between the total percentage of affected larvae and Se concentrations in both eggs and larvae ($\chi^2 = 68.9$ and 66.5, respectively, $p < 0.0001$; Table 3-9; Figures 3-6 & 3-7). Logit probabilities were used to estimate that 10, 25 and 50 percent of the population would become affected (edema and/or skeletal deformity) at *ca.* 15, 22, and 29 μg/g Se in larvae, respectively (Figure 3-6). The effective doses are nearly identical for Se in eggs (Figure 3-7).

Hypothesis testing of the percentage of developmental defects observed in larvae compared across exposure level and developmental stage revealed some differences (Table 3-10). Overall, the greatest difference between control and treatment groups was

in the total percentage of larvae affected (edema and/or skeletal deformities; two-way nested ANOVA following angular transformation, $p = 0.004$; Tukey HSD, $\alpha = 0.05$). Considered separately, both edema ($p = 0.037$) and skeletal deformities ($p = 0.002$) were observed in greater numbers in the treatment group (two-way nested ANOVA following angular transformation; Tukey HSD, $\alpha = 0.05$). Each type of skeletal deformity was also analyzed separately. Only scoliosis was found to appear more often in the treatment group (Wilcoxon Rank Sums, $p < 0.025$).

Microinjection

As with the maternal exposure experiment, developmental defects measured included edema and the skeletal deformities: lordosis, kyphosis, and scoliosis. For microinjection *study one*, logit analysis showed significant associations between the occurrence of edema ($\chi^2 = 42.7$) and skeletal deformities ($\chi^2 = 147$) and Se concentrations in larvae at the onset of larval development ($p < 0.0001$; Table 3-11; Figures 3-8 & 3-9). Logit probabilities were used to estimate that 25 and 50 percent of the population would incur edema at *ca.* 13 and 42 μg/g Se in larvae, respectively (Figure 3-8). Skeletal deformities would be expected in 15, 25 and 50 percent of the population at *ca.*10, 18, and 32 μg/g Se in larvae, respectively (Figure 3-9). As with the maternal experiment, the total measurement of affected larvae (edema and/or skeletal deformities) was used to reflect the overall impact on each experimental group. Logit probabilities were used to estimate that 25 and 50 percent of the population would become affected (edema and/or skeletal deformity) at *ca.* 7 and 17 μg/g Se in larvae, respectively ($\chi^2 = 202.8$, $p < 0.0001$;

Table 3-11; Figure 3-10). Approximately 500 larvae from one female were measured for developmental defects in *study one*.

For microinjection *study two*, logit analysis showed significant associations between the occurrence of edema ($\chi^2 = 132.3$) and skeletal deformities ($\chi^2 = 26.4$) and Se concentrations in larvae at the onset of larval development ($p < 0.0001$; Table 3-12; Figures 3-11 & 3-12). Logit probabilities were used to estimate that 15, 25 and 50 percent of the population would incur edema at *ca.* 11, 13 and 15 μg/g Se in larvae, respectively (Figure 3-11). Skeletal deformities would be expected in 15, 25 and 50 percent of the population at *ca.* 15, 17, and 21 μg/g Se in larvae, respectively (Figure 3-12). Logit probabilities were used to estimate that 15, 25 and 50 percent of the population would become affected (edema and/or skeletal deformity) at *ca.* 10, 12, and 15 μg/g Se in larvae, respectively ($\chi^2 = 122.7$, $p < 0.0001$; Table 3-12; Figure 3-13). Approximately 500 larvae from a total of two females were measured for developmental defects in *study two*.

Hypothesis testing of the percentage of developmental defects observed in yolk sac larvae compared across exposure levels revealed some differences (Table 3-13; *study two*). The high Se treatment showed significantly higher percentages of edema and lordosis (Rank F-Test, $p < 0.005$; Tukey HSD, $\alpha = 0.05$). Greater numbers of larvae with edema were observed in the low Se treatment compared to the medium Se treatment (one-way ANOVA following angular transformation, $p \leq 0.05$, Tukey HSD, $\alpha = 0.05$). A greater percentage of skeletal deformities in general (lordosis, scoliosis, or kyphosis) and of total affected larvae (edema and/or skeletal deformities) was observed in the high treatment group (one-way ANOVA following angular transformation, $p \leq 0.05$, Tukey

HSD, $\alpha = 0.05$). The control non-injected and sham-injected groups were not significantly different and were combined into one group for the above comparisons (Students t-test, $p > 0.4$). Similarly, the high, medium and low L-methionine data were combined into one group since they were not significantly different from each other (Students t-test, $p > 0.4$). Hypothesis comparisons of developmental effects were not possible in microinjection *study one* because only one progeny replicate was used.

Combined Maternal and Microinjection Data

Data from the maternal and microinjection experiments were combined to provide a more robust analysis of developmental defects following Se exposure during larval development. Logit analysis of the combined data showed significant associations between the occurrence of edema ($\chi^2 = 199.8$), skeletal deformities ($\chi^2 = 340.1$) and Se concentrations in larvae at the onset of larval development ($p < 0.0001$; Tables 3-7 – 3-9, 3-11 & 3-12; Figures 3-14 & 3-15). Logit probabilities were used to estimate that 15, 25 and 50 percent of the population would incur edema at *ca.* 18, 26 and 40 μg/g Se in larvae, respectively (Figure 3-14). Skeletal deformities would be expected in 15, 25 and 50 percent of the population at *ca.* 18, 23, and 34 μg/g Se in larvae, respectively (Figure 3-15). Logit probabilities were used to estimate that 15, 25 and 50 percent of the population would become affected (edema and/or skeletal deformity) at *ca.* 11, 15, and 22 μg/g Se in larvae, respectively ($\chi^2 = 543.2$, $p < 0.0001$; Figure 3-16). All Se values are from stage 36 larvae and represent exposures at the onset of larval development.

Mortality in Larvae

Maternal Exposure

Mortality in larvae was analyzed using Kaplan-Meier survival analysis. Approximately 14,000 larvae from a total of six females were monitored for survival. Data are categorized in two ways for the analysis: by maternal dietary exposure level and by high (*ca.* 21 μg/g), medium (*ca.* 9 μg/g) and low (*ca.* 2 μg/g) ranges of Se concentrations in eggs. Both approaches revealed significant differences in the probability of mortality over time (in days post-hatch; Table 3-14; Figure 3-17). When comparing between maternal dietary exposure levels, we find a probability of 0.03 (3 percent) of mortality in treatment larvae by the end of larval development (Kaplan-Meier, $p < 0.0001$). Comparison of the Kaplan-Meier distributions shows that the treatment group is significantly different from the control group (Student's t-test, $p < 0.0001$). However, when the data are categorized by the amount of Se in eggs, we observe a greater difference between groups and a probability of 0.08 (8 percent) of mortality in the high group (*ca.* 21 μg/g) by the end of larval development (Kaplan-Meier, $p < 0.0001$). Differences between these Kaplan-Meier distributions follow High (*ca.* 21 μg/g Se) >> Low (*ca.* 2 μg/g) > Medium (*ca.* 9 μg/g; Student's t-test, $p < 0.0001$).

Microinjection

Incidence of mortalities and the percentage of developmental effects observed within mortalities for the microinjection studies are shown in Table 3-15. Both microinjection studies show a statistically significant difference in the probability of larval mortality between exposure levels, over time (Kaplan-Meier, $p < 0.0001$; Figure 3-

18). In microinjection *study one*, the probability of larval mortality in low, medium, and high treatment groups by the end of larval development were approximately 0.35, 0.45 and 0.65, respectively (Kaplan-Meier, $p < 0.0001$). Comparison of the Kaplan-Meier distributions shows the relationship high (*ca.* 46 μg/g Se) > medium (*ca.* 22 μg/g Se) > low (*ca.* 16 μg/g Se) > control (*ca.* 3 μg/g Se; Student's t-test, $p \leq 0.008$). Approximately 500 larvae from one female were monitored for survival in *study one*.

In microinjection *study two*, the low and medium treatment groups are categorized as one group (low) due to indistinguishable Se concentrations. The probability of larval mortality in low and high treatment groups by the end of larval development was approximately 0.05 and 0.70, respectively (Kaplan-Meier, $p < 0.0001$; Table 3-15; Figure 3-18). Differences between Kaplan-Meier distributions follow high (*ca.* 17 μg/g Se) > low (*ca.* 9 μg/g Se) > control (*ca.* 6 μg/g Se; Student's t-test, $p < 0.001$). Approximately 500 larvae from a total of two females were monitored for survival in *study two*.

Combined Maternal and Microinjection Data

Table 3-16 summarizes percent mortality in white sturgeon larvae following Se exposure using the combined data of the maternal and microinjection studies. The combined data are categorized by Se concentrations in larvae at the start of larval development (*ca.* stage. 36; after hatching in maternal study; several hours post injection in microinjection studies). Kaplan-Meier survival analysis of combined mortality data shows a statistically significant difference between exposure categories ($p < 0.0001$; Figure 3-19). Differences between Kaplan-Meier distributions follow very high (*ca.* 46 μg/g Se) >> high (*ca.* 18 – 22 μg/g Se) = mid (*ca.* 12 – 16 μg/g Se) > low (*ca.* 8 – 10

μg/g Se) > control (*ca.* 2 – 7 μg/g Se; student's t-test, $p < 0.05$). The probability of mortality by the end of larval development in larvae exposed to 12 to 22 and 46 μg/g Se were approximately 0.15 to 0.29 and 0.65, respectively (Kaplan-Meier, $p < 0.0001$; student's t-test, $p < 0.05$).

Discussion

This work shows that white sturgeon are susceptible to Se-induced developmental toxicity at environmentally relevant levels. The observed effects of Se on larval development included edema, skeletal deformities and mortality. No impact on the size of larvae was observed.

Se maternal transport resulted in significant Se burdens persisting in developing larvae (Table 3-5). Se appears to be conserved throughout the yolk utilization period (Figure 3-1). Chapter two of this dissertation revealed that maternally transferred Se was predominantly in the form of yolk proteins. The incorporation of selenoproteins in larval tissue and increase of Se in circulation could both contribute to the conservation of Se during larval development. The excretory mechanisms are limited during the yolk sac phase of larval development in sturgeon, and the efficient mechanisms of gill excretion and glomerular filtration seem to be fully established only at the end of yolk absorption (Dettlaff *et al.* 1993; Wrobel 2003).

The microinjection studies were designed to mimic maternal transfer of Se. Microinjection of Se-L-Met into yolk sac larvae proved to be a reliable method to study the effects of excess Se on the development of this species. In microinjection *study one* we successfully targeted the exposure levels of 10, 20 and 40 μg/g Se in larvae (Table 3-

6; Figure 3-2). The observed increase of Se in control larvae between the start and end of the experiment was most likely due to different sample sizes (n = 60 and 10, respectively) and different analytical methods employed for the two analyses (ICP-AES and Fluorometry, respectively). In microinjection *study two*, the actual Se concentrations in microinjected larvae differed from the target exposure levels of 3, 9 and 27 µg/g Se, but essentially provided two levels of Se exposure (*ca.* 9 and 17 µg/g; Table 3-6; Figure 3-2). In both microinjection studies, we were unable to accurately obtain target exposures levels that were less than 10 µg/g apart. Small amounts of the compound may have diffused out of the embryo or larvae directly following injection, due to the water solubility of Se-L-Met. Overall, microinjection of white sturgeon larvae resulted in environmentally relevant levels of Se exposures. Actual exposure levels of Se in larvae ranged from *ca.* 8 to 47 µg/g (Table 3-6; Figure 3-2). The loss of Se between the start and end of the microinjection studies contradicts the findings of the maternal study. This may be due to the fact that Se in the yolk of maternally exposed larvae was protein bound while the microinjected larvae received Se in the form of free amino acids. The observed effects of Se on larval development included edema, skeletal deformities (lordosis, kyphosis and scoliosis), and mortality. Similar types of deformities were observed in the maternal exposure and microinjection studies. Developmental defects were low in the control groups of all studies (< 4%; Tables 3-10, 3-11 & 3-13). Exposure to *ca.* 10 µg/g Se led to developmental defects in 0 to 13 percent of maternally exposed larvae (T1 and T3; Table 3-10) and 5 to 13 percent of microinjected larvae (low and medium dose of *study two*; Table 3-13). Exposure to *ca.* 16 to 47 µg/g Se led to very high incidence of developmental defects in microinjected larvae (70 – 84%; Tables 3-11 & 3-13).

However, maternally exposed larvae in cohort two (T2) containing *ca.* 20 μg/g Se showed considerably less incidence of edema and/or skeletal defects (28% by the end of yolk absorption; Table 3-10), suggesting that seleno-amino acid injection produced more of an acute effect.

In both the maternal exposure and microinjection studies, developing larvae showed statistically significant relationships between Se concentrations in larvae and the occurrence of edema and skeletal deformities (Logit, $p < 0.0001$; Figures 3-4 – 3-16). Comparisons of defect occurrence rates between exposure groups (Tables 3-10 & 3-13) were less useful than logit analysis, which linked the Se exposure of each progeny cohort directly to the observed effects. Logit probability equations were used to estimate the median effective Se dose (ED_{50}) for each defect (Table 3-17). The term ED_X is used here to represent the level of Se content in developing larvae that leads to developmental defects in X percent of the tested population. The probability of developing either edema or skeletal deformities was used to estimate the overall impact on development of larvae exposed to excess Se. In general, the microinjected larvae appeared to be more sensitive to Se; however, many of the ED_X values are comparable between studies (Table 3-17). By combining the data of all three studies, it is estimated that larval Se concentrations of 11 and 22 μg/g will lead to edema and/or skeletal deformities in 15 and 50 percent of the population, respectively.

Mortality increased with increasing larval Se concentrations in both the maternal and microinjection studies (Tables 3-14 & 3-15; Figures 3-17 & 3-18). Microinjected larvae showed higher levels of mortality compared to larvae in the maternal exposure experiment. In the maternal study, 8 percent mortalities was observed with *ca.* 20 μg/g

Se exposure, but similar Se exposures were associated with 45 to 70 percent mortalities in the microinjection studies. Mortality was very low or absent in the control groups of all studies (< 2%; Tables 3-14 & 3-15). When considering the combined data from all experiments, the probabilities of mortality by the end of yolk absorption in larvae exposed to 12 to 22 and 46 μg/g Se were approximately 15 - 29 and 65 percent, respectively (Kaplan-Meier, $p < 0.0001$; student's t-test, $p < 0.05$; Figure 3-19).

There are certainly differences between the microinjection and maternal exposure studies. Mortality was significantly higher in treatment larvae from the microinjection experiment (10 – 70%) compared to the maternal exposure experiment (0 – 8.5%; Tables 3-14 & 3-15). Similarly, edema and/or skeletal deformities occurred at lower Se levels in microinjected larvae (ED_{50} = 14.5 – 16.8 μg/g Se) than in larvae exposed to Se-laden maternal yolk (ED_{50} = 28.8 μg/g Se; Table 3-17). The higher sensitivity of the microinjection larvae is likely due to the effects of injection stress, form of Se used (free amino acids in microinjection versus protein bound in maternal exposure), and the route of Se exposure (injected versus incorporated into yolk proteins). Microinjected larvae may have absorbed the free Se-L-Met very quickly. Conversely, the maternally exposed larvae absorbed protein-bound Se, as well as vital nutrients, as they digested yolk over time. This could have resulted in different rates of Se exposure, which may have led to the observed differences between these experiments. The timing of the production of a critical enzyme in Se detoxification (superoxide dismutase) has been shown to influence the sensitivity of larvae to Se toxicity (Palace *et al.* 2004). If greater levels of free Se-L-Met were absorbed prior to the production of key detoxifying enzymes or cofactors, then greater sensitivity would be expected. These suggestions remain speculative until

additional work elucidates the physiology of larval white sturgeon during exposure to excess Se. Nevertheless, both exposure routes led to adverse effects at Se concentrations exceeding *ca.* 11 to 15 µg/g in eggs and larvae. The very low control mortality and abnormality rates indicate that white sturgeon larvae tolerate microinjection well, and that microinjection of yolk sac larvae is a good model for investigating Se-induced toxicity in early life stages.

The observed effects on larvae agree with those described in several field and laboratory experiments. The most common types of developmental defects produced by Se toxicity are severe edema and various forms of terata, including spinal curvatures (Gillespie and Baumann 1986; Lemly 1993b; Woock *et al.* 1987). Holm *et al.* (2005) found 41.85 and 31.85 µg/g Se (converted from wet weight using 80% moisture) in the eggs of rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*) collected from seleniferous areas in Canada. These eggs produced larvae with edema and craniofacial and skeletal deformities. Gillespie and Baumann (1986) spawned female bluegills (*Lepomis macrochirus)* from a high Se reservoir with egg Se concentrations of 12 to 55 µg/g. The resulting larvae exhibited edema, deformities and complete mortality. Other field and laboratory studies have shown similar results in bluegill (Coyle *et al.* 1993; Hermanutz *et al.* 1992; Woock *et al.* 1987). Schultz and Hermanutz (1990) experimentally demonstrated that fathead minnow (*Pimephales promelas*) embryos containing *ca.* 19.5 µg/g Se (converted from wet weight using 80% moisture) resulted in larvae with edema and spinal deformities. Hamilton *et al.* (2002a) found that eggs from wild razorback suckers (*Xyrauchen texanus*) containing greater than 20 µg/g Se represented a high level of hazard to this endangered species.

Several possible mechanisms of Se toxicity have been described. Palace *et al.* (2004) demonstrated that selenomethionine exposure can lead to oxidative stress in fish larvae, which is known to cause edema and spinal curvatures. They linked superoxide production to methioninase enzyme activity and the ensuing metabolism of selenomethionine. Superoxide activity affects the embryo until significant production of superoxide dismutase provides a detoxification pathway. Individual variation in the expression of either of these enzymes could lead to different timing and frequencies of effects. Additionally, Se can be incorporated into biological molecules in place of sulfur because the two elements have similar chemical properties (Diplock 1976; Stadtman 1974). However, Se and sulfur bonds may have different strength and function in biological molecules (Reddy and Massaro 1983), thus some Se-substituted compounds may become unstable or dysfunctional (Lemly 1998; Stadtman 1974). Se can also inhibit protein synthesis (Vernie and Van Leewenhoekhuis 1987) and react with sulfhydryl groups of proteins or other molecules (Martin 1973), causing disruptions of histogenesis and deformities, which are a common effect of Se toxicity (Lemly 1998).

These results indicate that white sturgeon populations that bioaccumulate elevated Se may experience decreased recruitment. White sturgeon in San Francisco Bay are exposed to high levels of Se through their diet, evidenced by high Se levels in common prey of white sturgeon (Johns and Luoma 1988; Linville *et al.* 2002; White *et al.* 1988), as well as in sturgeon muscle, liver and eggs (Kroll and Doroshov 1991; Urquhart and Regalado 1991; White *et al.* 1987; White *et al.* 1988; White *et al.* 1989). White sturgeon sampled from San Francisco Bay-Delta between 1986 and 1990 contained Se at concentrations ranging from 9 to 30 μg/g in liver (n= 52) and 7 to 15 μg/g in muscle (n =

99; Urquhart and Regalado 1991; White *et al.* 1988). Very limited data are available on Se concentrations in the eggs of wild white sturgeon. Out of six sturgeon females sampled in the San Francisco Bay-Delta region in the 1990's, one contained eggs with 3 μg/g Se, four had eggs ranging from 8 to 12 μg/g Se and one female contained eggs with 29 μg/g Se (Kroll and Doroshov 1991). Recently, three white sturgeon captured from San Francisco Bay-Delta were found to have 7 to 20 μg/g Se in ovaries containing developing eggs (Doroshov Lab, UCD; unpublished data).

In the work described here (chapters 2 and 3) female sturgeon with liver Se concentrations of 11.0 and 11.6 μg/g produced larvae with 13 and 28 percent occurrence of developmental defects, respectively (Table 3-18). The remaining treatment female, that was successfully induced to spawn (T1), contained 8.7 μg/g Se in the liver and produced larvae that were free of developmental defects. In these limited data, maternal brood fish Se muscle concentrations appear to correspond with the incidence of developmental defects in progeny (Table 3-18). If there is a true relationship between muscle and egg Se levels, then white sturgeon populations could be monitored by analyzing non-lethal core samples of muscle.

Se levels in eggs and larvae provide an estimate of the direct exposure of Se to the developing larvae during yolk utilization. Such data probably provide the best prediction of Se-toxicity in developing larvae but are very difficult to obtain from wild populations, although naturally spawned eggs can be collected using spawning mats. The limited data available suggest that white sturgeon in the San Francisco Bay-Delta are carrying egg Se burdens that present a significant hazard to their progeny. Table 3-19 shows potential hazard threshold levels for Se in developing white sturgeon, based on both the maternal

and microinjection exposure experiments presented here. Larval Se concentrations of 3.4 and 8.2 µg/g are predicted to result in edema and/or skeletal deformities in 5 and 10 percent of developing larvae, respectively. This Se hazard threshold range is based on the observed development of *ca.* 1,000 maternally exposed larvae and 1,000 microinjected larvae, and the survival of *ca.* 14,000 maternally exposed larvae and 1,000 microinjected larvae during the course of these experiments. Egg Se levels could potentially be substituted for these larval threshold values, based on the conservation of Se observed throughout the early development in the maternal experiment (dry weight values only; Figure 3-1). Most of the wild sturgeon egg Se data from San Francisco Bay-Delta fall above this threshold range. This work indicates that Se toxicity is likely contributing to the reduced recruitment observed in the white sturgeon population of San Francisco Bay-Delta (California Department of Fish and Game Commission 2006; Schaffter and Kohlhorst 1999).

In summary, white sturgeon larvae exposed to Se by maternal transfer and microinjection demonstrated significant increases of mortality and abnormality rates (including edema and skeletal deformities) in larvae containing Se concentrations above *ca.* 11 to 15 µg/g. The larval Se exposures consisted of environmentally relevant Se levels and revealed defect and mortality rates similar to those observed in field and laboratory studies of Se toxicity in other fish. This work shows that Se toxicity is expected to cause significant impacts in white sturgeon populations that bioaccumulate elevated levels of Se. A hazard threshold range of *ca.* 3 to 8 µg/g Se in developing white sturgeon is suggested for this species.

Se content of experimental diets in maternal exposure experiment

Diet	Measured Total Selenium (μg/g)
Control	1.42 ± 0.03
Treatment	34.04 ± 0.82

Table 3-1. Selenium averages ± standard error (dw) are shown. Selenium was added to the diet as selenized yeast (Selenomax®, Ambi Inc.).

Key developmental stages in sturgeon

Stage 5 ***2nd cleavage***	The onset of the first cleavage is indicated by the appearance of a furrow in the animal pole of the fertilized egg. Due to the holoblastic cleavage and yolky eggs in sturgeon, the second cleavage furrow appears in the animal pole resulting in four incompletely divided cells sharing yolk and cytoplasm material. White sturgeon eggs reach the stage 5 about five hours post fertilization at 15.7 °C (Deng, 2000).
Stage 19 – 23 ***neurulation***	The neural folds begin to form around the head region (stage 19), extend to the trunk region (stage 20), and come close together (stage 21-22) to form a neural tube. By stage 23 the neurulation is completed and a suture-line between the two folds is clearly visible.
Stage 29 ***S-shaped heart***	The heart primordium forms as a straight tube, bends as a C-tube, and later as an S-shaped tube (stage 29). At stage 29 heart beat commences.
Stage 36 ***peak hatch***	At this stage, hatched yolk sac larvae are 10-11 mm in total length, darkly pigmented, and are relatively underdeveloped. They lack eye lenses, mouth, functional gills, and have continuous median fanfold.
Stage 40 ***pyloric sphincter formation***	At this stage a pyloric sphincter forms between the anterior portion (future stomach) and the posterior portion (future intestine) of the yolk sac. The intestinal portion is void of yolk material, but stomach is not differentiated and is filled with yolk.
Stage 45 ***yolk depletion***	At this stage all the fins and fin rays have differentiated. The mouth is well developed and equipped with teeth. Four barbells are located in the ventral side of the snout anterior to the mouth. The gill filaments project from the operculum. Stomach, liver, pancreas, and spleen are differentiated, and some fish had extruded the yolk plug and are ready to start exogenous feeding.

Table 3-2. Developmental staging following Dettlaff *et al*. (1993) for Russian sturgeon, with specific characteristics for white sturgeon described by Beer (1981) and Deng (2000).

Size of larvae from control and treatment female white sturgeon

Weight of Larvae During Development

	Control			*Treatment*		
	Mean Weight (mg)	**Standard Error**	**n**	**Mean Weight (mg)**	**Standard Error**	**n**
Stage 36	21.86	2.328	3	22.72	3.116	3
Stage 40	24.77	2.066	2	25.82	4.127	3
Stage 45	35.16	4.013	2	32.60	2.472	3

Length of Larvae During Development

	Control			*Treatment*		
	Mean Length (mm)	**Standard Error**	**n**	**Mean Length (mm)**	**Standard Error**	**n**
Stage 36	11.00	0.186	3	11.55	0.605	3
Stage 40	13.39	0.391	2	14.22	0.338	3
Stage 45	17.27	0.693	2	17.80	0.323	3

n=number of progeny cohorts

Table 3-3. Weight (mg) and length (mm) of larvae at three developmental stages. Larvae are progeny of female white sturgeon exposed to either control (*ca.* 1.4 μg/g) or treatment (*ca.* 34 μg/g) dietary Se for approximately six months during vitellogenesis. Approximately 30 larvae were measured for n number of progeny cohorts. No significant differences were detected between control and treatment larvae for any developmental stage (nested ANOVA or Rank F-Test; $p > 0.2$).

Size of larvae in Microinjection Study Two

	Weight (mg)	Length (mm)
Female 1	24.23 ± 1.82	12.48 ± 0.33
Female 3	17.66 ± 2.22	12.85 ± 0.35

Table 3-4a. Initial length and weight (mean ± standard deviation, n=15) of larvae from Female 1 and Female 3, used for microinjection study two. Newly hatched Female 1 larvae had significantly greater weight (Wilcoxon Rank Sums; $p < 0.0001$) but were shorter (ANOVA; $p = 0.006$) than Female 3 larvae.

	Weight (mg)	Length (mm)
Non-injected	42.55 ± 2.96^{b}	18.45 ± 0.39^{ab}
Sham-injected	42.28 ± 1.74^{b}	18.60 ± 0.50^{a}
L-Met Low *	45.04^{a}	18.57^{ab}
L-Met Med	41.76 ± 2.51^{b}	18.33 ± 0.23^{ab}
L-Met High	41.74 ± 1.00^{b}	18.09 ± 0.01^{bc}
Se-L-Met Low	42.49 ± 2.52^{b}	18.50 ± 0.27^{a}
Se-L-Met Med	42.44 ± 2.45^{b}	18.67 ± 0.30^{a}
Se-L-Met High §	37.87^{c}	17.56^{c}

Table 3-4b. Weight and length (mean ± standard error of the two progeny cohorts; 10 – 15 larvae per cohort) of larvae at stage 45 (yolk depletion) from treatment and control groups in microinjection study two. Within each measurement type, levels not connected by same letter are significantly different (nested ANOVA, $p < 0.0001$; Tukey HSD $\alpha = 0.05$).

*Sample included larvae from Female 1 only, which were initially higher in weight and shorter in length than larvae from Female 3.

§Sample included larvae from Female 3 only, which were initially lower in weight and longer in length than larvae from Female 1.

Selenium in developing white sturgeon progeny from female white sturgeon exposed to dietary selenium.

	Se μg/g ± Standard Error (n)		
Tissue	***Control***	***Treatment***	***Percent Moisture (Avg ± Standard Deviation)***
Egg	2.25 ± 0.326 (3)	12.4 ± 2.801 (4)‡	63.7 ± 4.1
Stg. 5 Embryo	2.32 ± 0.279 (3)	11.78 ± 2.711 (4)‡	75.7 ± 2.3
Stg. 21 Embryo	2.21 ± 0.304 (3)	8.92 ± 1.173 (3)‡	73.8 ± 2.2
Stg. 33 Embryo	1.95 ± 0.173 (3)	12.04 ± 2.98 (3)‡	79.5 ± 5.1
Stg. 36 Larvae	2.26 ± 0.295 (3)	12.58 ± 3.113 (3)‡	76.5 ± 1.1
Stg. 40 Larvae	2.34 ± 0.54 (2)	13.07 ± 3.875 (3)‡	84.5 ± 3.4
Stg. 45 Larvae	2.28 ± 0.315 (2)	13 ± 3.44 (3)‡	92.4 ± 1.5

Table 3-5. Se in developing white sturgeon progeny of females exposed to either control (*ca.* 1.4 μg/g) or treatment (*ca.* 34 μg/g) dietary Se for approximately six months during vitellogenesis. Data displayed as mean Se μg/g (dw) ± standard error (n = progeny cohorts; 60 – 90 larvae per cohort). ‡Significant difference from the corresponding control value (Student's t-test, $p < 0.0001$).

Selenium in developing white sturgeon exposed to seleno-L-methionine by microinjection

Study One

	Post-injection Se	Stage 45 Se
Non-injected	2.60	5.43 ± 0.14
Sham-injected	2.54	5.19 ± 0.17
Low	15.8	13.92 ± 1.03
Med	21.7	10.71 ± 0.34
High	46.6	8.99 ± 0.34

a)

Study Two

	Post-injection Se	Stage 40 Se	Stage 45 Se
Non-injected	6.35 ± 0.35 (2)	5.36 ± 0.3 (2)	6.27 ± 1.21 (2)
Sham-injected	5.89 ± 0.77 (2)	6.27 ± 0.04 (2)	5.81 ± 0.59 (2)
Low	8.74 ± 0.36 (2)	7.89 ± 0.15 (2)	9.46 ± 0.47 (2)
Med	8.96 ± 0.2 (2)	9.59 ± 0.34 (2)	8.51 ± 1.85 (2)
High	16.55 ± 1.62 (2)	17.61 ± 1.04 (2)	10.13 (1)

b)

Table 3-6. Selenium in developing white sturgeon exposed to seleno-L-methionine by microinjection. a) ***Microinjection Study One*** using progeny of one female white sturgeon. Whole larvae selenium content (µg/g, dw) one day post injection (stage *ca.* 36; ICP-AES analysis, n = *ca.* 45 larvae) and at the experimental endpoint (stage 45; Fluorometric analysis, n = 5 – 10 larvae, data displayed as µg/g Se ± standard error of sample duplicates). b) ***Microinjection Study Two*** using progeny of two female white sturgeon. Whole larvae selenium content one day post injection (stage *ca.* 36), mid-experiment (stage 40) and at the experimental endpoint (stage 45). All analysis by Fluorometry; data displayed as µg/g Se ± standard error of progeny replicates (n = progeny cohorts; 5 – 10 larvae per cohort). Statistical analysis presented in Figure 3-2. Percent moisture in stages 36, 40 and 45 was 69.4 ± 5.6, 84.6 ± 2.3 and 84.3 ± 2.9, respectively (average ± standard deviation).

Occurrence of edema in larvae from female white sturgeon exposed to dietary selenium shown by progeny cohort

	Control			Treatment		
	Cohort	Percent with Edema	Larval Se‡	Cohort	Percent with Edema	Larval Se‡
Stage 36	C3	0.00 (1)	2.43	T1	0.00 (1)	11.60
	C4	0.00 (1)	1.69	T2	0.00 (1)	18.40
	C5	0.00 (1)	2.67	T3	6.67 (1)	7.75
Stage 40	C4	0.00 (3)	1.80	T1	0.00 (3)	11.60
	C5	0.00 (3)	2.88	T2	4.44 ± 2.22 (3)	20.40
				T3	1.67 ± 1.67 (2)	7.22
Stage 45	C4	0.00 (3)	1.96	T1	0.00 (3)	12.00
	C5	0.00 (3)	2.59	T2	15.56 ± 1.11 (3)	19.40
				T3	0.00 (2)	7.61

Table 3-7. Percentage of larvae affected by edema for each progeny cohort of six female white sturgeon exposed to dietary selenium presented as average percentage of replicate samples ± standard error of replicates (number of replicate samples, containing 30 larvae each*). Data are shown for each of the larval developmental stages sampled. Logit analyses of the data is presented in Figure 3-4. ‡Selenium concentration shown in μg/g and based on dry weight. *Some replicate samples contained slightly less than 30 larvae.

Occurrence of skeletal deformities in larvae from female white sturgeon exposed to dietary selenium shown by progeny cohort

	Control			Treatment		
	Cohort	Percent with Skeletal Defects	Larval Se‡	Cohort	Percent with Skeletal Defects	Larval Se‡
Stage 36	C3	0.00 (1)	2.43	T1	0.00 (1)	11.60
	C4	0.00 (1)	1.69	T2	0.00 (1)	18.40
	C5	0.00 (1)	2.67	T3	10.00 (1)	7.75
Stage 40	C4	1.11 ± 1.11 (3)	1.80	T1	0.00 (3)	11.60
	C5	1.11 ± 1.11 (3)	2.88	T2	14.44 ± 1.11 (3)	20.40
				T3	8.33 ± 1.67 (2)	7.22
Stage 45	C4	0.00 (3)	1.96	T1	0.00 (3)	12.00
	C5	0.00 (3)	2.59	T2	21.11 ± 1.11 (3)	19.40
				T3	13.33 ± 3.33 (2)	7.61

Table 3-8. Percentage of larvae affected by skeletal deformities (lordosis, kyphosis, or scoliosis) for each progeny cohort of six female white sturgeon exposed to dietary selenium. Data shown as average percentage of replicate samples ± standard error of replicates (number of replicate samples, containing 30 larvae each*). Data are given for each of the larval developmental stages sampled. Logit analyses of the data is presented in Figure 3-5. ‡Selenium concentration shown in μg/g and based on dry weight. *Some replicate samples contained slightly less than 30 larvae.

Affected larvae (edema and/or skeletal deformities) of female white sturgeon exposed to dietary selenium shown by progeny cohort

	Control				Treatment			
	Cohort	Percent Affected[§]	Egg Se[‡]	Larval Se[‡]	Cohort	Percent Affected[§]	Egg Se[‡]	Larval Se[‡]
Stage 36	C3	0.00 (1)	2.46	2.43	T1	0.00 (1)	11.00	11.60
	C4	0.00 (1)	1.61	1.69	T2	0.00 (1)	20.50	18.40
	C5	0.00 (1)	2.68	2.67	T3	16.67 (1)	7.61	7.75
Stage 40	C4	1.11 ± 1.11 (3)	1.61	1.80	T1	0.00 (3)	11.00	11.60
	C5	1.11 ± 1.11 (3)	2.68	2.88	T2	18.89 ± 1.11 (3)	20.50	20.40
					T3	10.00 ± 0 (2)	7.61	7.22
Stage 45	C4	0.00 (3)	1.61	1.96	T1	0.00 (3)	11.00	12.00
	C5	0.00 (3)	2.68	2.59	T2	27.78 ± 2.94 (3)	20.50	19.40
					T3	13.33 ± 3.33 (2)	7.61	7.61

Table 3-9. Percentage of larvae affected by edema and/or skeletal deformities for each progeny cohort of six female white sturgeon exposed to dietary selenium. Data are shown as average percentage of replicate samples ± standard error of replicates (number of replicate samples, 30 larvae each*). Data are given for each of the larval developmental stages sampled. Logit analyses of the data are presented in Figures 3-6 and 3-7.

[§]Larvae counted only once, even if both edema and skeletal defect occurred.
[‡]Selenium concentration shown in μg/g and based on dry weight.
*Some replicate samples contained slightly less than 30 larvae.

Occurrence of developmental defects in larvae from female white sturgeon exposed to dietary selenium

	Control				***Treatment***			
	Stage				**Stage**			
	36	**40**	**45**	**Combined Stages**	**36**	**40**	**45**	**Combined Stages**
Edema	0 ± 0 (3)	0 ± 0 (2)	0 ± 0 (2)	0 ± 0 (3)	2.22 ± 2.22 (3)	2.04 ± 1.3 (3)	5.19 ± 5.19 (3)	3.15 ± 1.75 (3)$^{\delta}$
Lordosis	0 ± 0 (3)	0 ± 0 (2)	0 ± 0 (2)	0 ± 0 (3)	1.11 ± 1.11 (3)	0.37 ± 0.37 (3)	0.37 ± 0.37 (3)	0.62 ± 0.38 (3)
Kyphosis	0 ± 0 (3)	0.56 ± 0.56 (2)	0 ± 0 (2)	0.16 ± 0.16 (3)	0 ± 0 (3)	4.26 ± 3.46 (3)	1.85 ± 1.85 (3)	2.04 ± 1.29 (3)
Scoliosis	0 ± 0 (3)	0.56 ± 0.56 (2)	0 ± 0 (2)	0.16 ± 0.16 (3)	2.22 ± 2.22 (3)	2.96 ± 1.96 (3)	8.52 ± 4.27 (3)$^{\phi}$	4.57 ± 1.80 (3)$^{\phi}$
Skeletal ‡	0 ± 0 (3)	1.11 ± 0 (2)	0 ± 0 (2)	0.32 ± 0.20 (3)	3.33 ± 3.33 (3)	7.59 ± 4.19 (3)$^{\delta}$	11.48 ± 6.16 (3)$^{\delta}$	7.47 ± 2.63 (3)$^{\delta}$
Total Affected §	0 ± 0 (3)	1.11 ± 0 (2)	0 ± 0 (2)	0.32 ± 0.20 (3)	5.56 ± 5.56 (3)	9.63 ± 5.46 (3)$^{\delta}$	13.7 ± 8.02 (3)$^{\delta}$	9.63 ± 3.43 (3)$^{\delta}$

Table 3-10. Percentage of larvae affected by edema, lordosis, kyphosis, and scoliosis for progeny of female white sturgeon that were exposed to dietary selenium. Data shown as average percentage of cohort replicates ± standard error of cohort replicates (number of cohort replicates*). Data are shown for each of the larval developmental stages sampled. ‡Grouped category of lordosis, kyphosis, and scoliosis. §Larvae with edema and/or skeletal deformities (larvae counted only once, even if both effects occurred). $^{\delta}$Significant difference from control value (angular transformed, two-way nested ANOVA, $p < 0.05$; Tukey HSD, $\alpha = 0.05$). $^{\phi}$Significant difference from control value (Wilcoxon Rank Sums, $p < 0.05$). *Each cohort replicate represents progeny of one female. Cohort replicates for stage 36 contained 25 – 30 larvae each. Cohort replicates for stages 40 and 45 contained 60 – 90 larvae each.

Occurrence of developmental defects in larvae exposed to seleno-L-methionine

Microinjection Study One

	Control (Non-Injected) (2.6 µg/g Se*)	Sham-Injected (2.54 µg/g Se*)	Low Dose (15.8 µg/g Se*)	Med Dose (21.7 µg/g Se*)	High Dose (46.6 µg/g Se*)
Edema	0 ± 0 (1)	0 ± 0 (1)	63.12 ± 0.96 (1)	49.98 ± 4.44 (1)	35.64 ± 6.18 (1)
Lordosis	0 ± 0 (1)	3.95 ± 2.61 (1)	17.48 ± 6.73 (1)	30.61 ± 5.3 (1)	40.6 ± 5.23 (1)
Kyphosis	0 ± 0 (1)	0 ± 0 (1)	9.7 ± 2.56 (1)	9.18 ± 1.78 (1)	23.76 ± 1.72 (1)
Scoliosis	0 ± 0 (1)	0 ± 0 (1)	0 ± 0 (1)	8.17 ± 3.67 (1)	1.99 ± 0.98 (1)
Skeletal ‡	0 ± 0 (1)	3.95 ± 2.61 (1)	27.17 ± 7.95 (1)	47.97 ± 7.35 (1)	66.33 ± 7.72 (1)
Total Affected §	0 ± 0 (1)	3.95 ± 2.61 (1)	76.69 ± 4.86 (1)	83.66 ± 3.67 (1)	78.21 ± 7.72 (1)

Table 3-11. Percentage of larvae affected by edema, lordosis, kyphosis, and scoliosis from ***Microinjection Study One***. Larvae were microinjected with a varying seleno-L-methionine after hatching. Data shown as average percentage ± standard error of three replicate tanks (number of cohort replicates♦). Data span early larval development (stages 36 – 45).

‡Grouped category of lordosis, kyphosis, and scoliosis.
§ Larvae with edema and/or skeletal deformities (larvae counted only once, even if both effects occurred).
♦Each cohort replicate represents progeny of one female. Each of the three replicate tanks contained *ca.* 35 larvae.
*Se exposure at the beginning of larval development (stage 36; Table 3-6).

Occurrence of edema in larvae from *microinjection study two* shown by progeny cohort

	Progeny F1				Progeny F3			
	Se µg/g*	Percent Edema	Percent Skeletal‡	Percent Total Affected§	Se µg/g*	Percent Edema	Percent Skeletal‡	Percent Total Affected§
Control (non-injected)	6.70	0.00	3.33	3.33	6.00	0.00	0.00	0.00
Sham-injected	6.66	0.00	0.00	0.00	5.12	0.00	0.00	0.00
Low Se	9.10	8.00	8.00	12.00	8.38	13.33	3.33	13.33
Medium Se	8.77	0.00	3.45	3.45	9.16	0.00	6.67	6.67
High Se	18.17	80.00	30.00	80.00	14.94	55.17	13.79	58.62

Table 3-12. Percentage of larvae with developmental defects for each progeny cohort of ***Microinjection Study Two***, used for logit analyses (Figures 3-11 – 3-13). Data span early larval development (stages 36 – 45) and represent approximately 30 larvae per treatment.

‡Selenium concentration shown in µg/g and based on dry weight.

*Selenium concentration represents the microinjected dose as measured post injection. Se values are shown in µg/g and based on dry weight.

‡ Grouped category of lordosis, kyphosis, and scoliosis.

§Larvae with edema and/or skeletal deformities (larvae counted only once, even if both effects occurred). Average data of the two cohorts are shown in Table 3-13.

Occurrence of developmental defects in larvae exposed to seleno-L-methionine

Microinjection Study Two

	Control[♦] (6 μg/g Se*)	L-Met Control[•] *(Se not analyzed)*	Low Dose (8.7 μg/g Se*)	Med Dose (9 μg/g Se*)	High Dose (16.6 μg/g Se*)
Edema	0 ± 0 (2)$^{a\delta}$	0 ± 0 (2)$^{a\delta}$	10.91 ± 3.64 (2)$^{b\delta}$	0 ± 0 (2)$^{a\delta}$	67.8 ± 13.56 (2)$^{c\delta}$
Lordosis	0 ± 0 (2)$^{a\delta}$	0 ± 0 (2)$^{a\delta}$	3.64 ± 0 (2)$^{b\delta}$	3.39 ± 0 (2)$^{ab\delta}$	15.25 ± 5.08 (2)$^{b\delta}$
Kyphosis	0.85 ± 0.85 (2)	1.19 ± 1.19 (2)	0 ± 0 (2)	0 ± 0 (2)	1.69 ± 1.69 (2)
Scoliosis	0 ± 0 (2)	1.19 ± 1.19 (2)	1.82 ± 1.82 (2)	1.69 ± 1.69 (2)	5.08 ± 1.69 (2)
Skeletal [‡]	0.85 ± 0.85 (2)^{a}	2.38 ± 2.38 (2)^{ab}	5.45 ± 1.82 (2)^{ab}	5.08 ± 1.69 (2)^{ab}	22.03 ± 8.47 (2)^{b}
Total Affected [§]	0.85 ± 0.85 (2)^{a}	2.38 ± 2.38 (2)^{a}	12.73 ± 1.82 (2)^{a}	5.08 ± 1.69 (2)^{a}	69.49 ± 11.86 (2)^{b}

Table 3-13. Percentage of larvae affected by edema, lordosis, kyphosis, and scoliosis from ***Microinjection Study Two***. Larvae were microinjected with a varying seleno-L-methionine after hatching. Data shown as average percentage of cohort replicates ± standard error of cohort replicates (number of cohort replicates[θ]). Data span early larval development (stages 36 – 45). Within the same row, levels not connected by same letter are significantly different (one-way ANOVA following angular transformation, $p \leq 0.05$, Tukey HSD, $\alpha = 0.05$). Data are not compared between rows.

[δ]Rank F-Test, $p < 0.005$; multiple comparisons by Tukey HSD, $\alpha = 0.05$.
[θ]Each cohort replicate represents progeny of one female and contained approximately 30 larvae per treatment.
[♦]Combined control non-injected and sham-injected samples (not significantly different; students t-test, $p > 0.4$).
[•]Combined L-Met control samples (not significantly different; students t-test, $p > 0.4$).
[‡]Grouped category of lordosis, kyphosis, and scoliosis.
[§]Larvae with edema and/or skeletal deformities (larvae counted only once, even if both effects occurred).
*Se exposure at the beginning of larval development (stage 36; Table 3-6).

Mortality in larvae from female white sturgeon exposed to dietary selenium

	Egg Se	*Stg 40* Percent Mortality	*Stg 45* Percent Mortality
Control	2.15 ± 0.54 (2)	0.0 ± 0.0 (2)	0.27 ± 0.0 (2)
Treatment	13.04 ± 4.72 (3)	0.04 ± 0.04 (3)	2.95 ± 2.74 (3)

a)

	Egg Se	*Stg 40* Percent Mortality	*Stg 45* Percent Mortality
Low	2.15 ± 0.54 (2)	0.0 ± 0.0 (2)	0.27 ± 0.0 (2)
Med	9.31 ± 1.70 (2)	0.07 ± 0.07 (2)	0.22 ± 0.12 (2)
High	20.5 (1)	0.0 (1)	8.42 (1)

b)

Table 3-14. Percent mortality in larvae from female white sturgeon exposed to dietary selenium. Mortality data presented as percent mortality ± standard error (number of progeny cohorts; 1,000 – 1,500 larvae per cohort). Egg selenium data are presented as Se μg/g (dw) ± standard error (number of progeny cohorts). a) Percent mortality by exposure levels. b) The same data but categorized by low, medium and high selenium concentration in eggs. Statistical analyses of these mortality data are presented in Figure 3-17.

			Effects as Percentage of Mortalities			
Study One	**Se (µg/g; dw)**	**Percent Mortality**	**Edema**	**Spinal Curvature**	**Edema and Spinal Curvature**	**Edema and/or Spinal Curvature**
Control (non-injected)	2.60	0.0%	0.00%	0.00%	0.00%	0.00%
Sham-injected	2.54	0.0%	0.00%	0.00%	0.00%	0.00%
Low Se	15.8	41.0%	4.76%	13.33%	13.33%	31.43%
Medium Se	21.7	46.7%	9.52%	27.62%	8.57%	45.71%
High Se	46.6	67.6%	4.76%	39.05%	22.86%	66.67%
Study Two						
Control (non-injected)	6.35 ± 0.35 (2)	0 ± 0 (2)	0 ± 0 (2)	0 ± 0 (2)	0 ± 0 (2)	0 ± 0 (2)
Sham-injected	5.89 ± 0.77 (2)	1.67 ± 1.67 (2)	0 ± 0 (2)	0 ± 0 (2)	0 ± 0 (2)	0 ± 0 (2)
L-Met Low	*not analyzed*	0 ± 0 (2)	0 ± 0 (2)	0 ± 0 (2)	0 ± 0 (2)	0 ± 0 (2)
L-Met Med	*not analyzed*	0 ± 0 (2)	0 ± 0 (2)	0 ± 0 (2)	0 ± 0 (2)	0 ± 0 (2)
L-Met High	*not analyzed*	1.67 ± 1.67 (2)	0 ± 0 (2)	1.67 ± 1.67 (2)	0 ± 0 (2)	1.67 ± 1.67 (2)
Low Se	8.74 ± 0.36 (2)	10.00 ± 0 (2)	6.67 ± 3.33 (2)	1.67 ± 1.67 (2)	1.67 ± 1.67 (2)	10 ± 0 (2)
Medium Se	8.97 ± 0.2 (2)	3.34 ± 3.34 (2)	0 ± 0 (2)	3.35 ± 3.35 (2)	0 ± 0 (2)	3.35 ± 3.35 (2)
High Se	16.56 ± 1.62 (2)	70.00 ± 16.67 (2)	46.7 ± 3.33 (2)	0 ± 0 (2)	20 ± 10 (2)	66.67 ± 13.33 (2)

Table 3-15. Percent mortality in larvae exposed to selenium via microinjection studies. Mortality data presented as percent mortality ± standard error (number of progeny cohorts). *Study one* included one progeny cohort (n = 100 larvae per treatment), while *study two* included two progeny cohorts (n = 30 larvae per each cohort). Selenium exposure is represented by concentrations in subsamples of larvae several hours following microinjection (*ca.* stage 36). Se data are presented as µg/g Se (dw) ± standard error (number of progeny cohorts). The percentage of defects observed in mortalities are displayed as percent defect ± standard error (number of progeny cohorts). Statistical analyses of these mortality data are presented in Figure 3-18.

Combined mortality in larvae from maternal exposure and microinjection studies

Grouped Exposure Categories	Average Percent Mortality
Control (~2 – 7 μg/g Se)	0.44 ± 0.31 (5)
Low (~8 – 10 μg/g Se)	5.35 ± 2.25 (5)
Mid (~12 – 16 μg/g Se)	31.54 ± 16.01 (3)
High (~18 – 22 μg/g Se)	47.25 ± 22.59 (3)
Very High (~46 μg/g Se)	67.62 (1)

Table 3-16. Percent mortality in larvae from maternal and microinjection studies of Se exposure in developing white sturgeon. Mortality data presented as percent mortality ± standard error (number of progeny cohorts). The combined data are categorized by the Se concentrations in larvae at the start of larval development (*ca.* stage. 36; after hatching in maternal study; several hours post injection in microinjection studies; values in dry weight). Statistical analyses of combined mortality data are presented in Figure 3-19.

Probabilities of development defects in larvae containing excess Se

	Skeletal Deformities			
	ED_{15}	**ED_{25}**	**ED_{50}**	**Logit Equation**
Maternal Exposure	20.0	24.4	31.9	$P(\text{skeletal deformity}) = 1/(1+e^{(-(-4.64 + 0.145 * (\text{Larval Se ug/g}))})$
Microinjection Study One	10.3	18.3	32.3	$P(\text{skeletal deformity}) = 1/(1+e^{(-(-2.54 + 0.08 * (\text{Larval Se ug/g}))})$
Microinjection Study Two	14.7	17.2	21.4	$P(\text{skeletal deformity}) = 1/(1+e^{(-(-5.59 + 0.26 * (\text{Larval Se ug/g}))})$
Combined Data	17.6	23.5	33.7	$P(\text{skeletal deformity}) = 1/(1+e^{(-(-3.63 + 0.11 * (\text{Larval Se ug/g}))})$

	Edema			
	ED_{15}	**ED_{25}**	**ED_{50}**	**Logit Equation**
Maternal Exposure	22.6	25.3	29.9	$P(\text{edema}) = 1/(1+e^{(-(-7.12 + 0.238 * (\text{Larval Se ug/g}))})$
Microinjection Study One	*	13.5	41.8	$P(\text{edema}) = 1/(1+e^{(-(-1.62 + 0.04 * (\text{Larval Se ug/g}))})$
Microinjection Study Two	11.5	12.8	14.9	$P(\text{edema}) = 1/(1+e^{(-(-7.62 + 0.51 * (\text{Larval Se ug/g}))})$
Combined Data	17.7	25.8	39.8	$P(\text{edema}) = 1/(1+e^{(-(-3.12 + 0.08 * (\text{Larval Se ug/g}))})$

	Total Affected (Edema and/or Skeletal Deformity)			
	ED_{15}	**ED_{25}**	**ED_{50}**	**Logit Equation**
Maternal Exposure**	17.9	21.9	28.8	$P(\text{affected larvae}) = 1/(1+e^{(-(-4.58 + 0.159 * (\text{Larval Se ug/g}))})$
Microinjection Study One	0.5	6.5	16.8	$P(\text{affected larvae}) = 1/(1+e^{(-(-1.79 + 0.11 * (\text{Larval Se ug/g}))})$
Microinjection Study Two	10.7	12.1	14.5	$P(\text{affected larvae}) = 1/(1+e^{(-(-6.56 + 0.45 * (\text{Larval Se ug/g}))})$
Combined Data	11.1	15.2	22.2	$P(\text{affected larvae}) = 1/(1+e^{(-(-3.48 + 0.16 * (\text{Larval Se ug/g}))})$

Table 3-17. Probabilities of developmental defects in larvae containing elevated Se levels at the beginning of larval development. Probabilities calculated from logit equations ($p < 0.0001$; Figures 3-4 – 3-16). ED_X represents the level of Se in larvae (μg/g, dw) expected to cause a particular effect in X percent of the population. Se concentrations are from larvae at the beginning of larval development (stage 36). Defects were measured throughout the period of yolk utilization (*ca.* first 10 days post-hatch). Data from each experiment is shown both separately and combined. *Could not be calculated. **Nearly identical to probabilities based on Se in eggs.

Se (μg/g; dw) in adult tissues and their progeny related to incidence of developmental defects

Exposure	Fish/ Progeny ID	Liver	Muscle	Ovarian	Egg	Larvae‡	Percent Developmental Defects*
Control	C3	1.33	1.28	1.49	2.46	2.43	0δ
Control	C4	0.80	1.22	1.65	1.61	1.69	0
Control	C5	2.16	1.48	1.38	2.68	2.67	0
Treatment	T1	8.72	9.93	6.53	11.00	11.6	0
Treatment	T2	11.60	15.30	13.20	20.50	18.4	27.78 ± 2.94
Treatment	T3	11.00	11.10	5.74	7.61	7.75	13.33 ± 3.33

Table 3-18. Se concentrations (μg/g; dw) in liver, muscle, ovarian tissue, and eggs from female white sturgeon exposed to either control (*ca.* 1.4 μg/g) or treatment (*ca.* 34 μg/g) dietary Se for approximately six months during vitellogenesis (described in Chapter 2). Se concentration and occurrence of developmental defects are shown for the progeny of each female (n = 25 – 30 larvae for stage 36; n = 60 - 90 larvae for stages 40 and 45).

.

‡Newly hatched larvae (stage 36)
*Occurrence of edema and/or skeletal deformities at the end of yolk sac development
δData from stage 36 only, due to very low hatch

Potential Hazard Threshold Levels for Se in developing white sturgeon larvae

	ED_{05}		ED_{10}	
	Se µg/g (dry wt)	**Se µg/g (wet wt)***	**Se µg/g (dry wt)**	**Se µg/g (wet wt)***
Edema	2.2	0.6	11.8	3.2
Skeletal Deformities	6.4	1.7	13.3	3.6
Edema and/or Skeletal Deformities	3.4	0.9	8.2	2.2

Table 3-19. Estimated probabilities of developmental defects in larvae from both maternal and microinjection experiments. ED_X represents the level of Se in stage 36 larvae expected to cause a particular effect in X percent of the population (Logit, $p < 0.0001$; Table 3-17). These effect levels are presented as potential hazard threshold levels for Se in newly hatched white sturgeon larvae. Defects were measured throughout the period of yolk utilization (*ca.* first 10 days post-hatch). Egg Se levels could potentially be substituted for the stage 36 Se thresholds suggested here since maternally transferred Se was conserved throughout early development in the maternal experiment (dry wt; Figure 3-1). *Estimated using the average percent moisture of stage 36 larvae from both studies, 73% ± 5.3 (average ± standard deviation).

Selenium in developing progeny of female white sturgeon exposed to dietary selenium

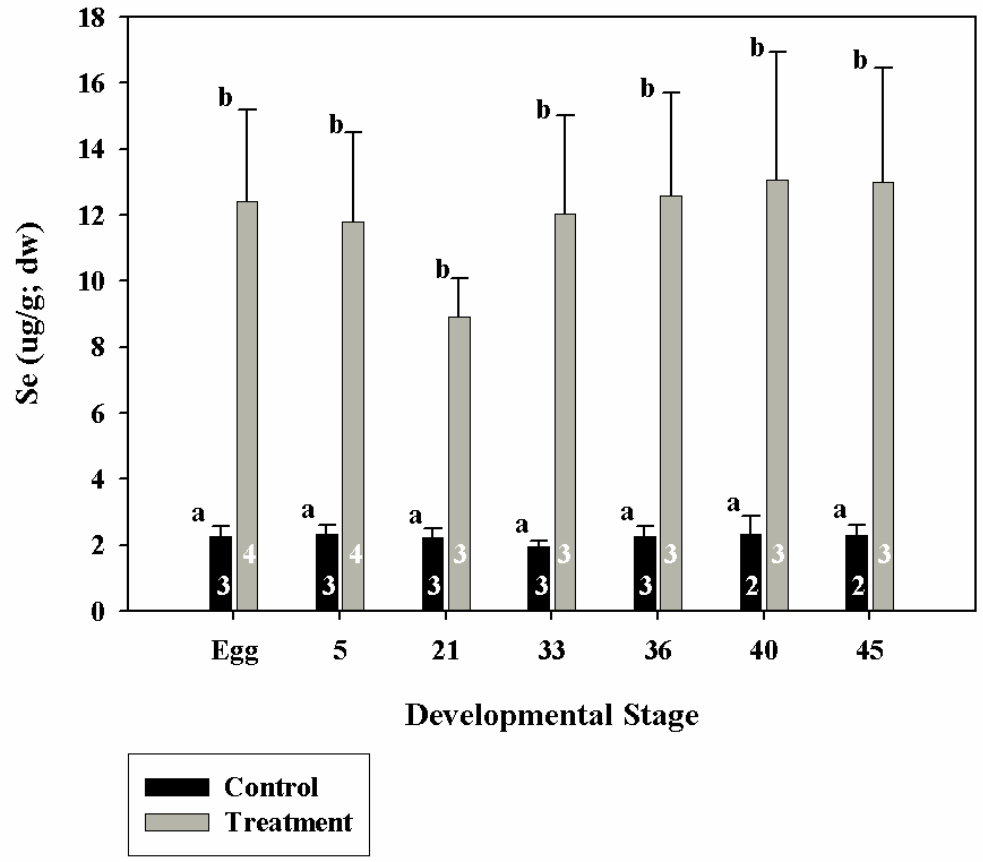
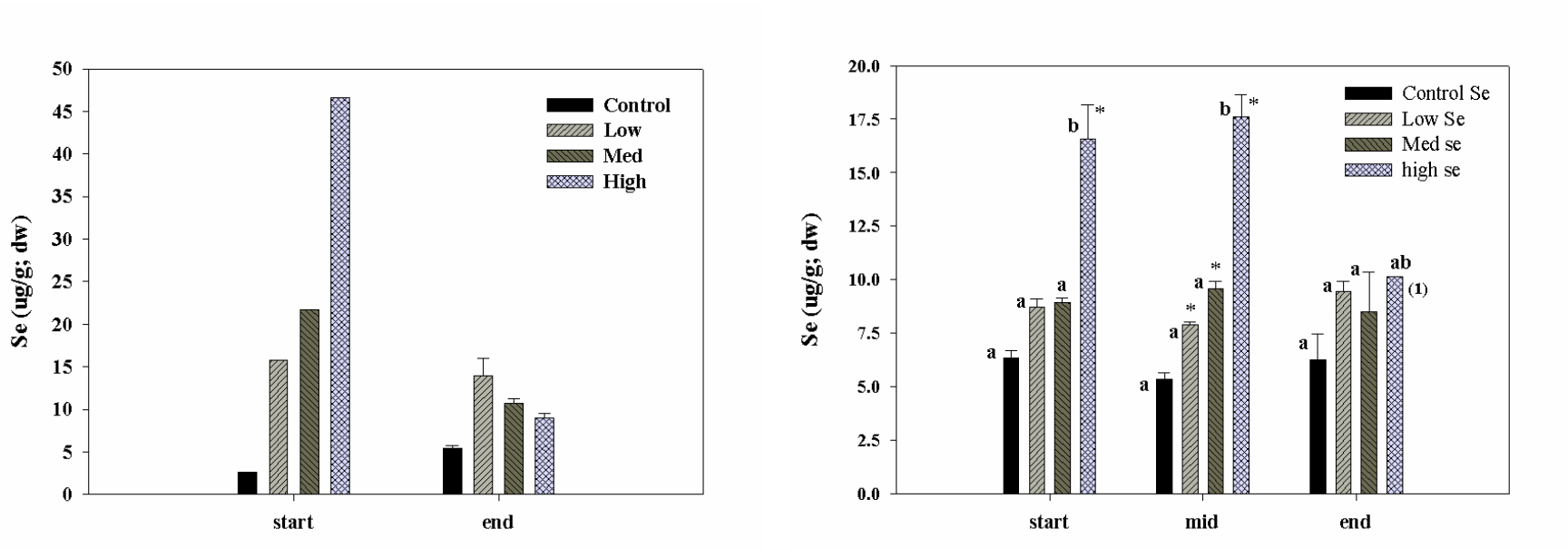


Figure 3-1. Selenium concentrations in eggs and developing embryos and larvae from white sturgeon females exposed to dietary selenium. Selenium is presented as mean Se µg/g (dw) ± standard error. The number of cohorts from individual maternal sturgeon are presented in each bar. Each cohort sample contained approximately 30 individuals. Groups not connected by the same letter are significantly different (lognormal, two-way nested ANOVA, $p < 0.0001$; multiple comparisons with Tukey HSD, $\alpha = 0.05$). Data are also presented in Table 3- 5.

Selenium in developing white sturgeon exposed to seleno-L-methionine by microinjection



a) Study One **b) Study Two**

Figure 3-2. Selenium concentrations in developing white sturgeon larvae following microinjection of seleno-L-methionine. **a)** ***Microinjection Study One*** using progeny of one female white sturgeon. Whole larvae selenium content at the start (one day post injection, stage *ca.* 36; ICP-AES analysis; n = *ca.* 45 larvae) and end of the experiment (stage 45, Fluorometry analysis, n = 5 – 10 larvae, data displayed as µg/g Se ± standard error of sample duplicates). **b)** ***Microinjection Study Two*** using progeny of two female white sturgeon. Whole larvae selenium content at the start (one day post injection, stage *ca.* 36), middle (stage 40) and at the experimental endpoint (stage 45). All analysis by Fluorometry; n = 5 – 10 larvae; data displayed as µg/g Se ± standard error of progeny replicates. All values from Study Two include two progeny replicates except the end, High treatment. Groups not connected by the same letter are significantly different (two-way, nested ANOVA, $p < 0.0001$; Tukey HSD, $\alpha = 0.05$). *Value is significantly different from control (ANOVA $p < 0.002$; Dunnets $\alpha = 0.05$). Data are also presented in Table 3- 6.

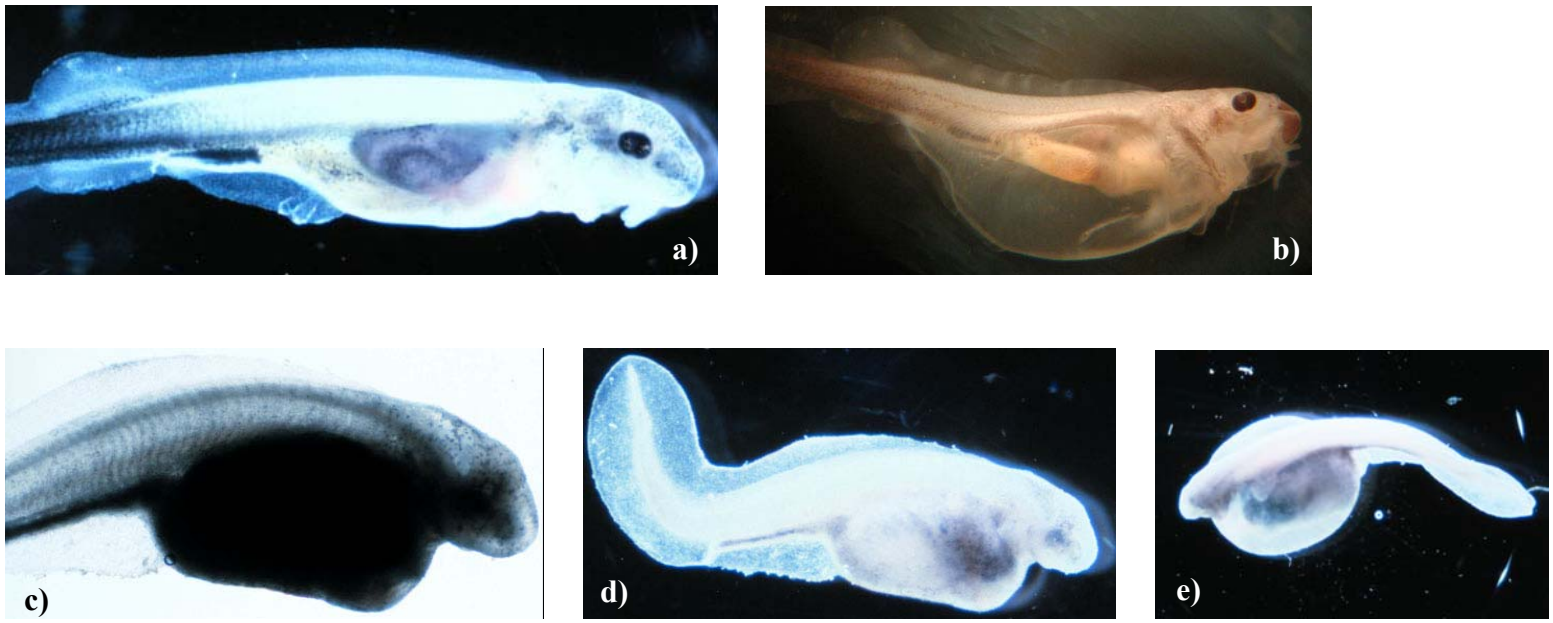
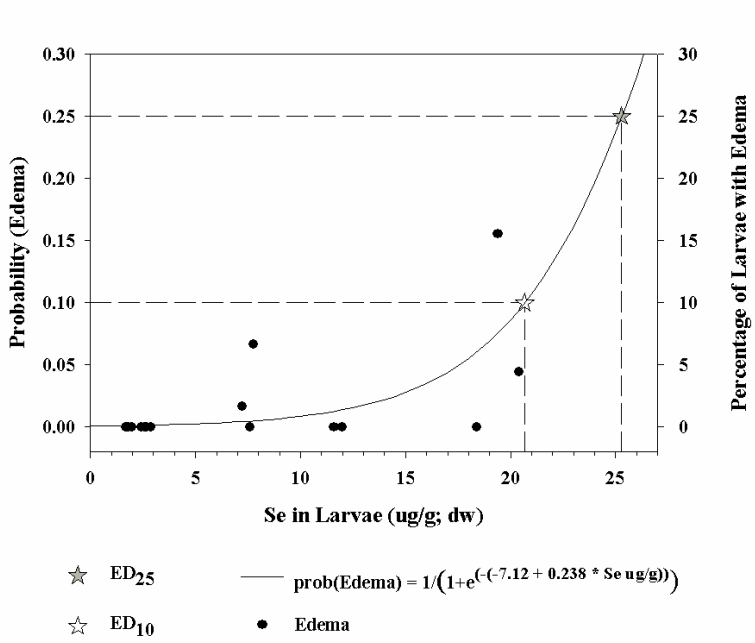


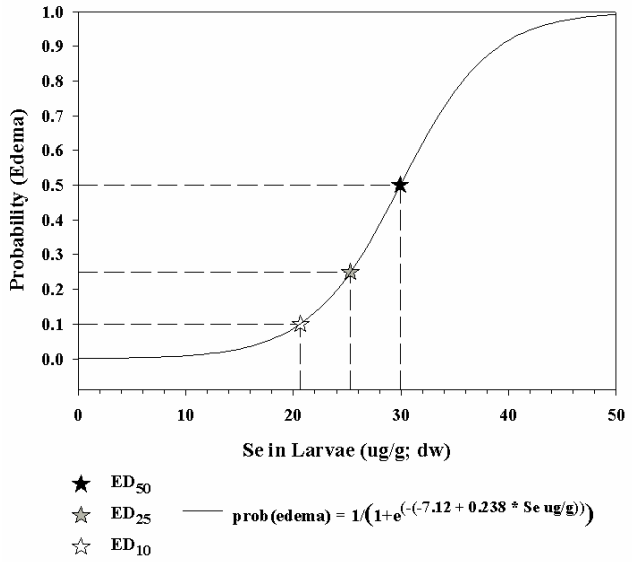
Figure 3-3. Developmental defects observed in white sturgeon during larval development. a) A normal larvae. b) Larvae with edema (overt swelling due to fluid accumulation). c) Larvae showing kyphosis (dorsoventral curvature of anterior region). d) Larvae showing lordosis (concave dorsoventral curvature of posterior region). e) Larvae with scoliosis (lateral skeletal curvature) and edema.

Progeny of selenium-exposed white sturgeon

larvae with edema related to larval selenium



(a)



(b)

Figure 3-4. Percentage of larvae with edema related to selenium concentrations in larvae (µg/g; dw). Larvae were progeny of adult white sturgeon females exposed to dietary selenium. Data includes three progeny cohorts from the control group and three from the treatment group. Data for three larval development stages are included (stages 36, 40 and 45). Logit analysis shows a significant association between selenium concentrations in larvae and the occurrence of defects ($\chi^2 = 39.7$, $p < 0.0001$; prob*(edema)* = $1/(1+e^{(-(-7.12 + 0.238 * (\text{Se in Larvae ug/g}))})$). (a) The right y-axis shows the actual percentage of affected larvae in samples containing 30 – 90 larvae each. The left y-axis shows the probability of larvae to develop edema at a given larval selenium concentration. (b) ED_{50} (29.90 µg/g), ED_{25} (25.28 µg/g) and ED_{10} (20.67 µg/g) values were calculated from the logit equation.

Progeny of selenium-exposed white sturgeon

larvae with skeletal deformities related to larval selenium

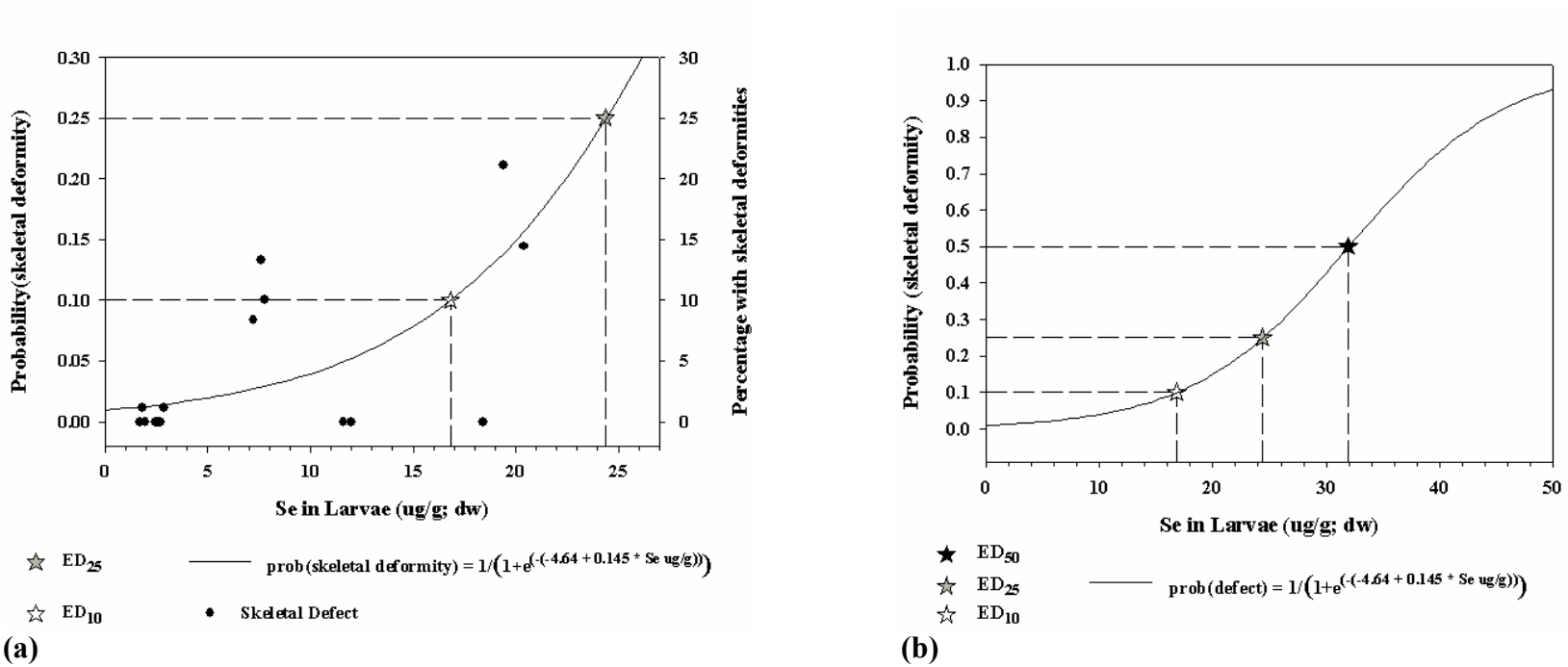
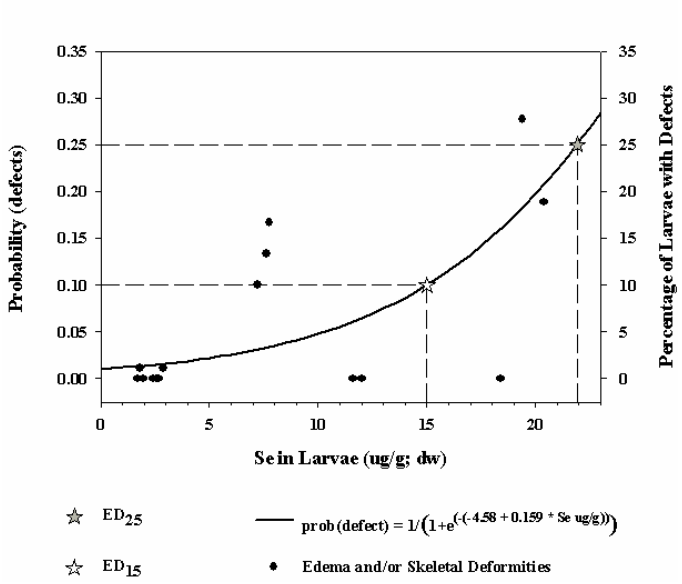


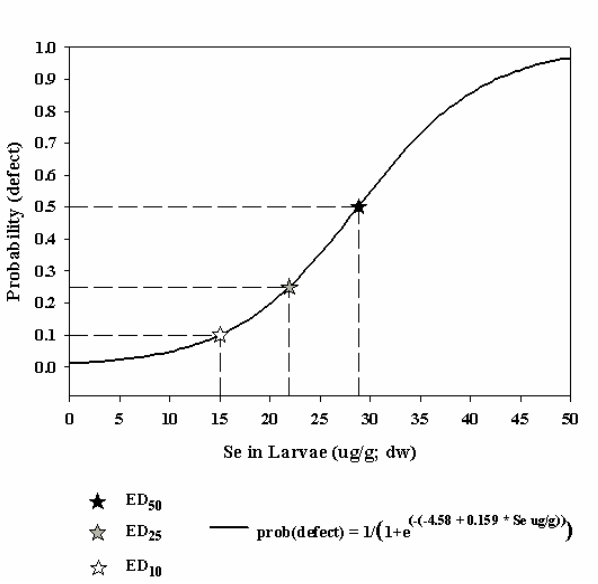
Figure 3-5. Percentage of larvae with skeletal deformities related to selenium concentrations in larvae (µg/g; dw). Larvae were progeny of adult white sturgeon females exposed to dietary selenium. Data includes three progeny cohorts from the control group and three from the treatment group. Data for three larval development stages are included (stages 36, 40 and 45). Logit analysis shows a significant association between selenium concentrations in larvae and the occurrence of defects ($\chi^2 = 46.3$, $p < 0.0001$; prob*(skeletal deformity)* = $1/(1+e^{(-(-4.64 + 0.145 * (\text{Se in Larvae ug/g}))})$). (a) The right y-axis shows the actual percentage of larvae with skeletal deformities in samples containing 30 – 90 larvae each. The left y-axis shows the probability of larvae to develop skeletal deformities at a given larval selenium concentration. (b) ED_{50} (31.92 µg/g), ED_{25} (24.37 µg/g) and ED_{10} (16.81 µg/g) values were calculated from the logit equation.

Progeny of selenium-exposed white sturgeon

larvae with edema and/or skeletal defects related to larval selenium



(a)

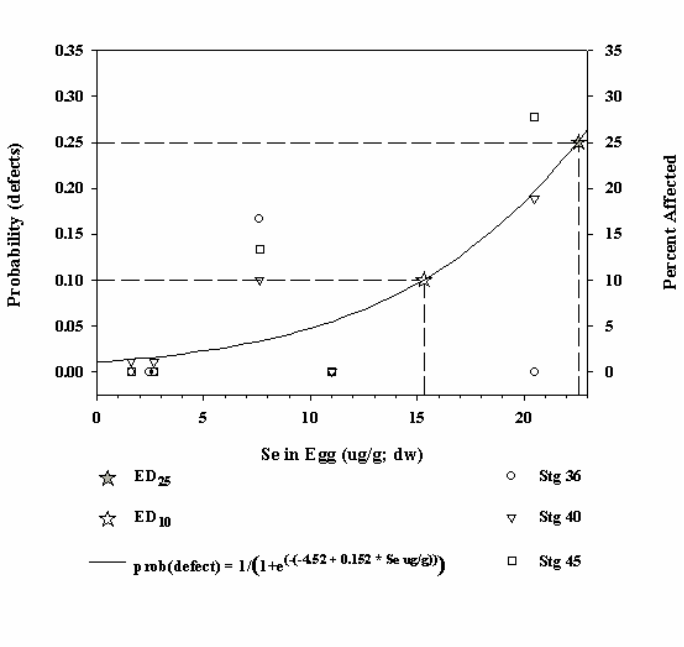


(b)

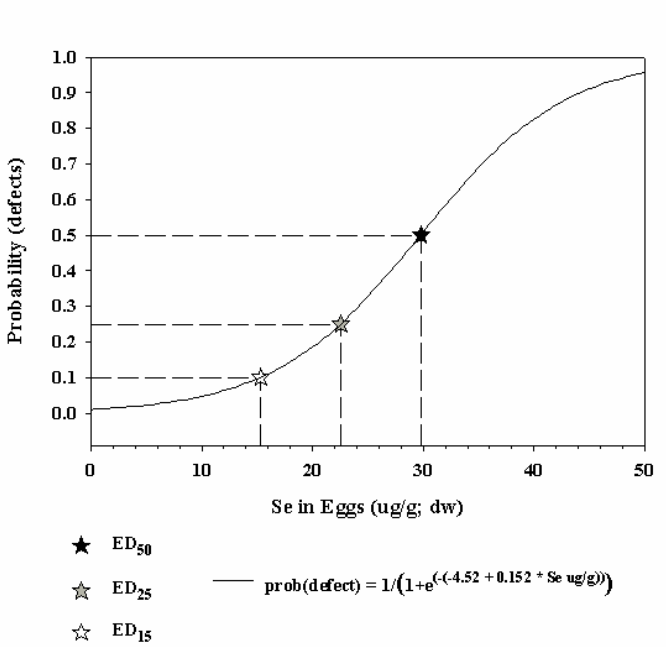
Figure 3-6. Percentage of larvae with edema and/or skeletal deformities related to selenium concentrations in larvae (µg/g; dw). Larvae were progeny of adult white sturgeon females exposed to dietary selenium. Data includes three progeny cohorts from the control group and three from the treatment group. Data for three larval development stages are included (stages 36, 40 and 45). Logit analysis shows a significant association between selenium concentrations in larvae and the occurrence of defects ($\chi^2 = 66.5$, $p < 0.0001$; prob*(defects)* = $1/(1+e^{(-(-4.58 + 0.159 * (\text{Se in Larvae ug/g}))})$). (a) The right y-axis shows the actual percentage of affected larvae in samples containing 30 – 90 larvae each. The left y-axis shows the probability of larvae to develop edema and/or skeletal deformities at a given larval selenium concentration. (b) ED_{50} (28.83 µg/g), ED_{25} (21.92 µg/g) and ED_{10} (15.01 µg/g) values were calculated from the logit equation.

Progeny of selenium-exposed white sturgeon

larvae with edema and/or skeletal defects related to egg selenium



(a)



(b)

Figure 3-7. Percentage of larvae with edema and/or skeletal deformities related to selenium concentrations in eggs (μg/g; dw). Larvae were progeny of adult white sturgeon females exposed to dietary selenium. Data includes three progeny cohorts from the control group and three from the treatment group. Data for three larval development stages are included (stages 36, 40 and 45). Logit analysis shows a significant association between selenium concentrations in larvae and the occurrence of defects ($\chi^2 = 68.9$, $p < 0.0001$; prob*(defects)* = $1/(1+e^{(-(-4.52 + 0.152 * (\text{Se in Eggs ug/g}))})$). (a) The right y-axis shows the actual percentage of affected larvae in samples containing 30 – 90 larvae each. The left y-axis shows the probability of larvae to develop edema and/or skeletal deformities at a given selenium concentration in eggs. (b) ED_{50} (29.81 μg/g), ED_{25} (22.56 μg/g) and ED_{10} (15.32 μg/g) values were calculated from the logit equation.

White sturgeon larvae following microinjection of seleno-L-methionine

Occurrence of edema related to larval selenium exposure

Microinjection Study One

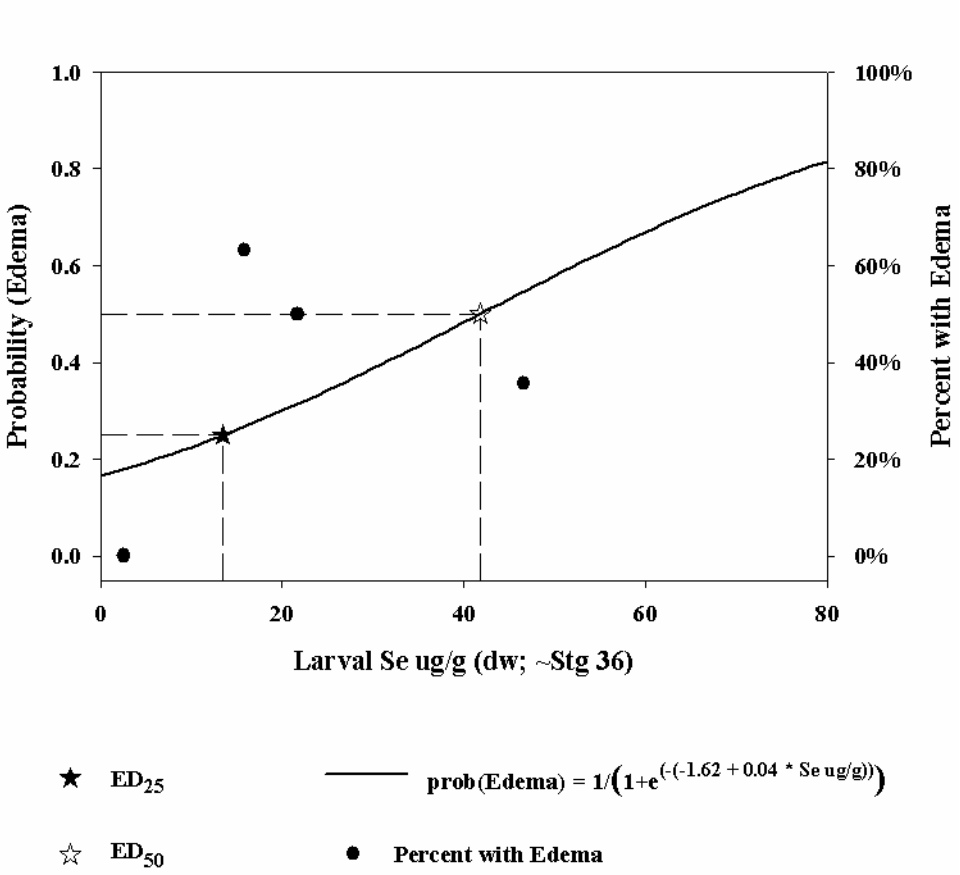


Figure 3-8. Percentage of larvae with edema related to selenium concentrations in larvae (μg/g; dw) at the start of larval development (*ca.* stage 36). Larvae were microinjected with seleno-L-methionine in ***microinjection study one***. Data includes one progeny cohort and spans early larval development (*ca.* stages 36 – 45). Logit analysis shows a significant association between selenium concentrations in larvae and the occurrence of defects ($\chi 2 = 42.7$, $p < 0.0001$; prob(*edema*) = $1/(1+e^{(-(-1.62 + 0.04 * (Se\ in\ Larvae\ ug/g))})$). The left y-axis shows the probability of larvae to develop edema at a given larval selenium concentration, as determined by logit analysis. The right y-axis shows the actual percentage of larvae with edema in samples containing *ca.* 100 larvae each. ED_{50} (41.8 μg/g) and ED_{25} (13.5 μg/g) values were calculated from the logit equation.

White sturgeon larvae following microinjection of seleno-L-methionine

Occurrence of skeletal deformities related to larval selenium exposure

Microinjection Study One

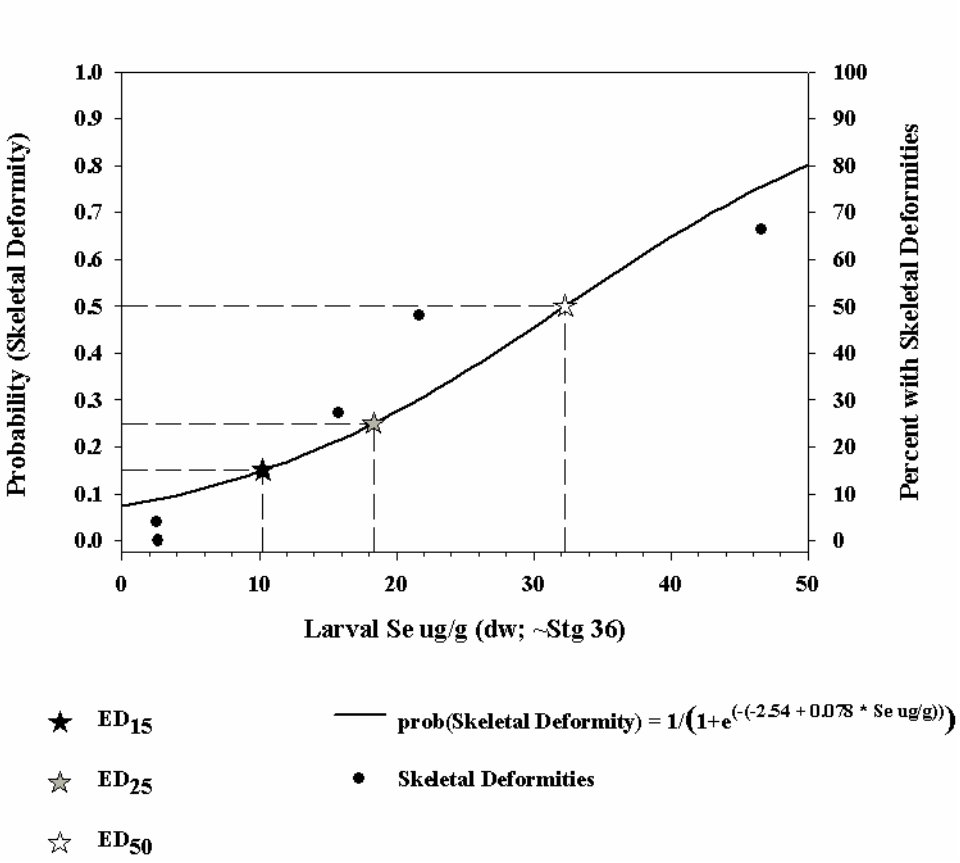


Figure 3-9. Percentage of larvae with skeletal deformities related to selenium concentrations in larvae (μg/g; dw) at the start of larval development (*ca.* stage 36). Larvae were microinjected with seleno-L-methionine in ***microinjection study one***. Data includes one progeny cohort and spans early larval development (*ca.* stages 36 – 45). Logit analysis shows a significant association between selenium concentrations in larvae and the occurrence of defects ($\chi 2 = 147$, $p < 0.0001$; prob(*skeletal deformity*) = $1/(1+e^{(-(-2.54 + 0.08 * (\text{Se in Larvae ug/g}))})$). The left y-axis shows the probability of larvae to develop skeletal deformities at a given larval selenium concentration, as determined by logit analysis. The right y-axis shows the actual percentage of larvae with skeletal deformities in samples containing *ca.* 100 larvae each. ED_{50} (32.3 μg/g), ED_{25} (18.3 μg/g) and ED_{15} (10.3 μg/g) values were calculated from the logit equation.

White sturgeon larvae following microinjection of seleno-L-methionine

Occurrence of skeletal deformities and/or edema related to larval selenium exposure

Microinjection Study One

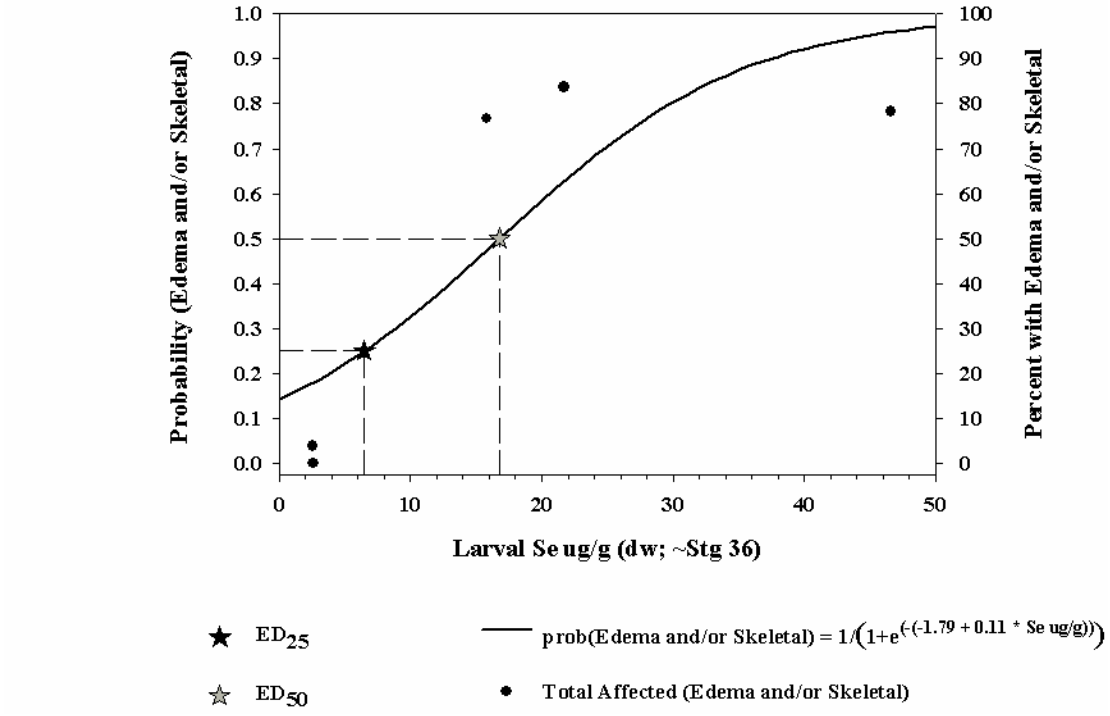


Figure 3-10. Percentage of larvae with edema and/or skeletal deformities related to selenium concentrations in larvae (µg/g; dw) at the start of larval development (*ca.* stage 36). Larvae were microinjected with seleno-L-methionine in ***microinjection study one***. Data includes one progeny cohort and spans early larval development (*ca.* stages 36 – 45). Logit analysis shows a significant association between selenium concentrations in larvae and the occurrence of defects ($\chi 2 = 202.8$, $p < 0.0001$; prob(*defects*) = $1/(1+e^{(-(-1.79 + 0.11 * (Se\ in\ Larvae\ ug/g))})$). The left y-axis shows the probability of larvae to develop edema and/or skeletal deformities at a given larval selenium concentration, as determined by logit analysis. The right y-axis shows the actual percentage of affected larvae in samples containing *ca.* 100 larvae each. ED_{50} (16.8 µg/g) and ED_{25} (6.5 µg/g) values were calculated from the logit equation.

White sturgeon larvae following microinjection of seleno-L-methionine

Occurrence of edema related to larval selenium exposure

Microinjection Study Two

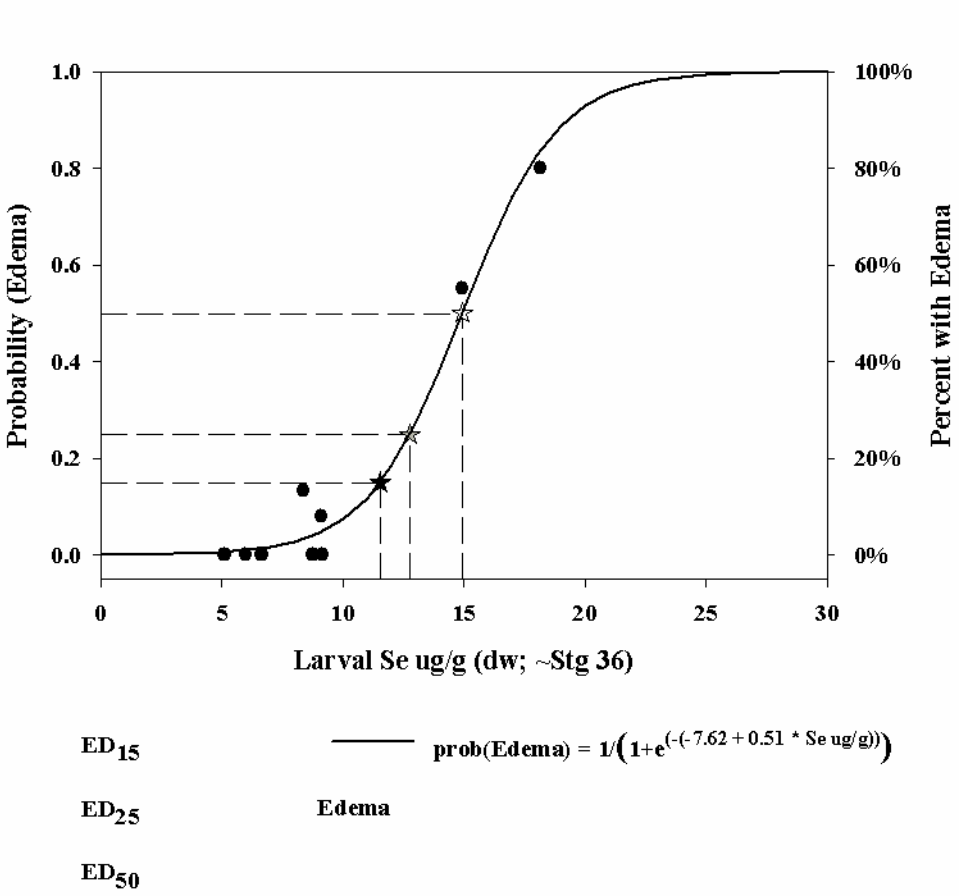


Figure 3-11. Percentage of larvae with edema related to selenium concentrations in larvae (μg/g; dw) at the start of larval development (*ca.* stage 36). Larvae were microinjected with seleno-L-methionine in ***microinjection study two***. Data includes two progeny cohorts and spans early larval development (*ca.* stages 36 – 45). Logit analysis shows a significant association between selenium concentrations in larvae and the occurrence of edema ($\chi 2 = 132.3$, $p < 0.0001$; prob(*edema*) = $1/(1+e^{(-(-7.62 + 0.51 * (\text{Se in Larvae ug/g}))})$). The left y-axis shows the probability of larvae to develop edema at a given larval selenium concentration, as determined by logit analysis. The right y-axis shows the actual percentage of larvae with edema in samples containing *ca.* 30 larvae each. ED_{50} (14.9 μg/g), ED_{25} (12.8 μg/g) and ED_{15} (11.5 μg/g) values were calculated from the logit equation.

White sturgeon larvae following microinjection of seleno-L-methionine

Occurrence of skeletal deformities related to larval selenium exposure

Microinjection Study Two

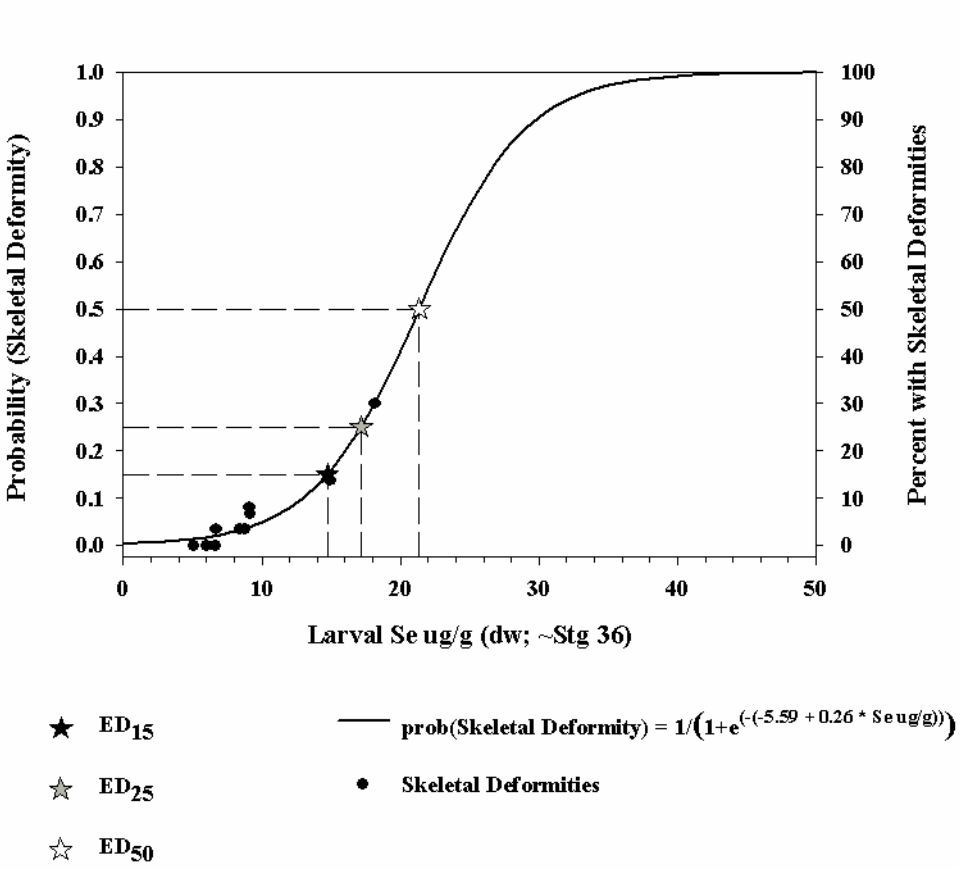


Figure 3-12. Percentage of larvae with skeletal deformities related to selenium concentrations in larvae (μg/g; dw) at the start of larval development (*ca.* stage 36). Larvae were microinjected with seleno-L-methionine in ***microinjection study two***. Data includes two progeny cohorts and spans early larval development (*ca.* stages 36 – 45). Logit analysis shows a significant association between selenium concentrations in larvae and the occurrence of skeletal deformities ($\chi 2 = 26.4$, $p < 0.0001$; prob(*skeletal deformity*) = $1/(1+e^{(-(-5.59 + 0.26 * (\text{Se in Larvae ug/g}))})$. The left y-axis shows the probability of larvae to develop skeletal deformities at a given larval selenium concentration, as determined by logit analysis. The right y-axis shows the actual percentage of larvae with skeletal deformities in samples containing *ca.* 30 larvae each. ED_{50} (21.4 μg/g), ED_{25} (17.2 μg/g) and ED_{15} (14.7 μg/g) values were calculated from the logit equation.

White sturgeon larvae following microinjection of seleno-L-methionine

Occurrence of skeletal deformities and/or edema related to larval selenium exposure

Microinjection Study Two

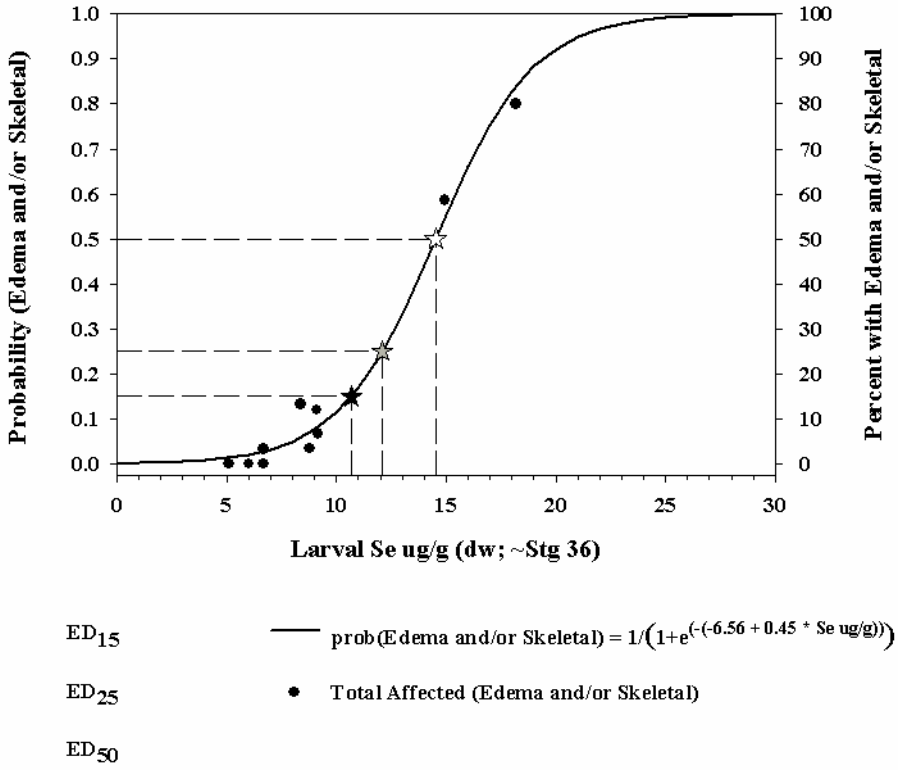


Figure 3-13. Percentage of larvae with edema and/or skeletal deformities related to selenium concentrations in larvae (μg/g; dw) at the start of larval development (*ca.* stage 36). Larvae were microinjected with seleno-L-methionine in ***microinjection study two***. Data includes two progeny cohorts and spans early larval development (*ca.* stages 36 – 45). Logit analysis shows a significant association between selenium concentrations in larvae and the occurrence of defects ($\chi 2 = 122.7$, $p < 0.0001$; prob(*defects*) = $1/(1+e^{(-(-6.56 + 0.45 * (Se in Larvae ug/g))})$). The left y-axis shows the probability of larvae to develop edema and/or skeletal deformities at a given larval selenium concentration, as determined by logit analysis. The right y-axis shows the actual percentage of affected larvae in samples containing *ca.* 30 larvae each. ED_{50} (14.5 μg/g), ED_{25} (12.1 μg/g) and ED_{15} (10.7 μg/g) values were calculated from the logit equation.

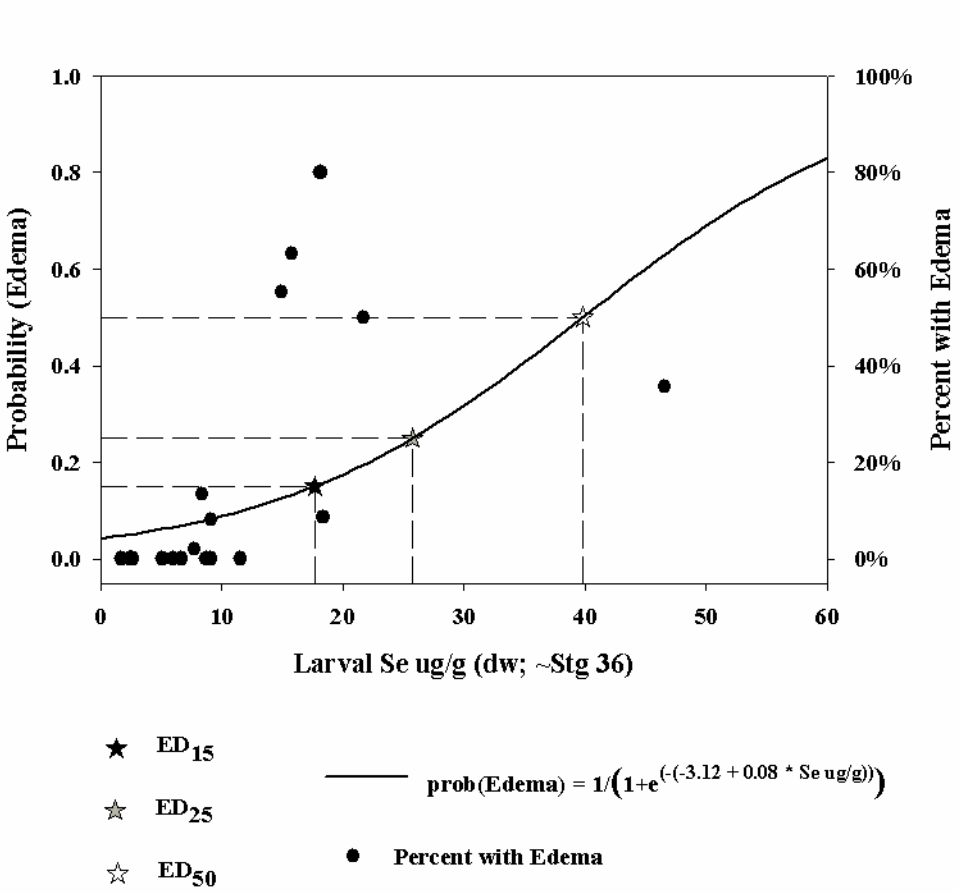


Figure 3-14. Percentage of larvae with edema related to selenium concentrations in larvae (µg/g; dw) at the start of larval development (*ca.* stage 36). Plot includes all data from the maternal exposure and microinjection experiments. Data includes nine progeny cohorts and spans early larval development (*ca.* stages 36 – 45). Logit analysis shows a significant association between selenium concentrations in larvae and the occurrence of edema ($\chi 2 = 199.8$, $p < 0.0001$; prob(*edema*) = $1/(1+e^{(-(-3.12 + 0.08 * (Se\ in\ Larvae\ ug/g))})$). The left y-axis shows the probability of larvae to develop edema at a given larval selenium concentration, as determined by logit analysis. The right y-axis shows the actual percentage of larvae with edema in samples containing *ca.* 30 - 200 larvae each. ED_{50} (39.8 µg/g), ED_{25} (25.8 µg/g) and ED_{15} (17.7 µg/g) values were calculated from the logit equation.

White sturgeon larvae following selenium exposure

Occurrence of skeletal deformities related to larval selenium exposure

Combined Data from Maternal and Microinjection Experiments

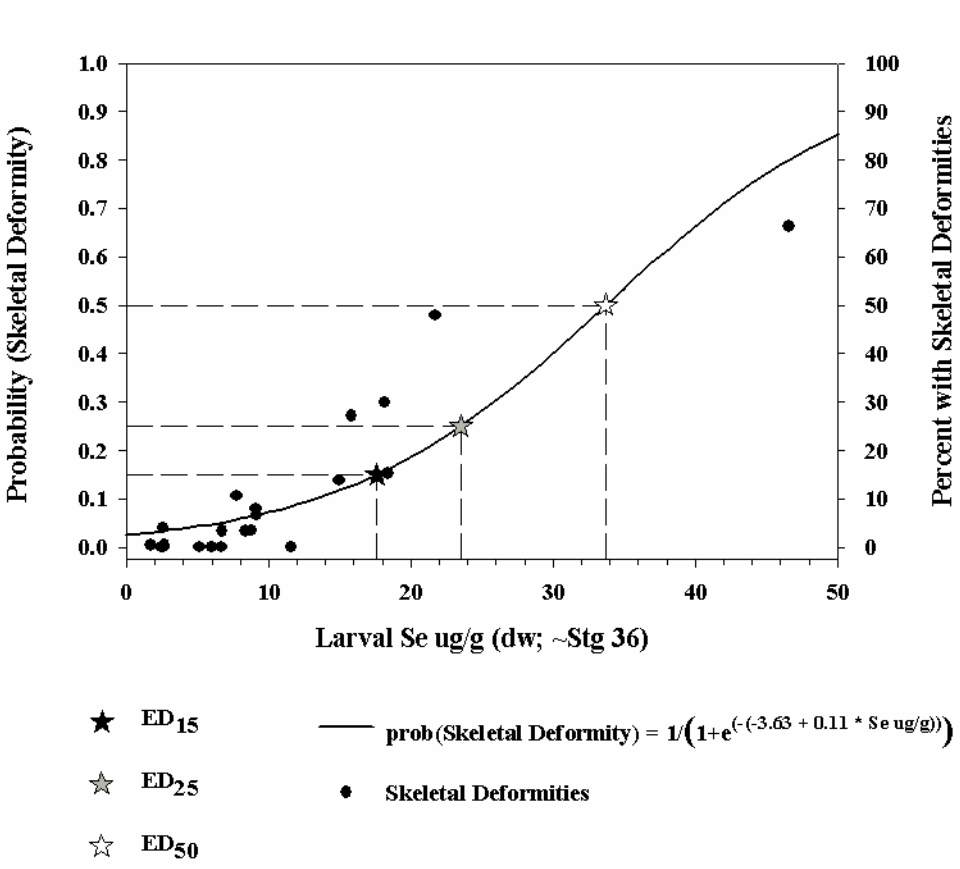


Figure 3-15. Percentage of larvae with skeletal deformities related to selenium concentrations in larvae (μg/g; dw) at the start of larval development (*ca.* stage 36). Plot includes all data from the maternal exposure and microinjection experiments. Data includes nine progeny cohorts and spans early larval development (*ca.* stages 36 – 45). Logit analysis shows a significant association between selenium concentrations in larvae and the occurrence of skeletal deformities ($\chi 2 = 340.1$, $p < 0.0001$; prob(*skeletal deformity*) = $1/(1+e^{(-(-3.63 + 0.11 * (\text{Se in Larvae ug/g}))})$. The left y-axis shows the probability of larvae to develop skeletal deformities at a given larval selenium concentration, as determined by logit analysis. The right y-axis shows the actual percentage of larvae with skeletal deformities in samples containing *ca.* 30 - 200 larvae each. ED_{50} (33.7 μg/g), ED_{25} (23.5 μg/g) and ED_{15} (17.6 μg/g) values were calculated from the logit equation.

White sturgeon larvae following selenium exposure

Occurrence of skeletal deformities and/or edema related to larval selenium exposure

Combined Data from Maternal and Microinjection Experiments

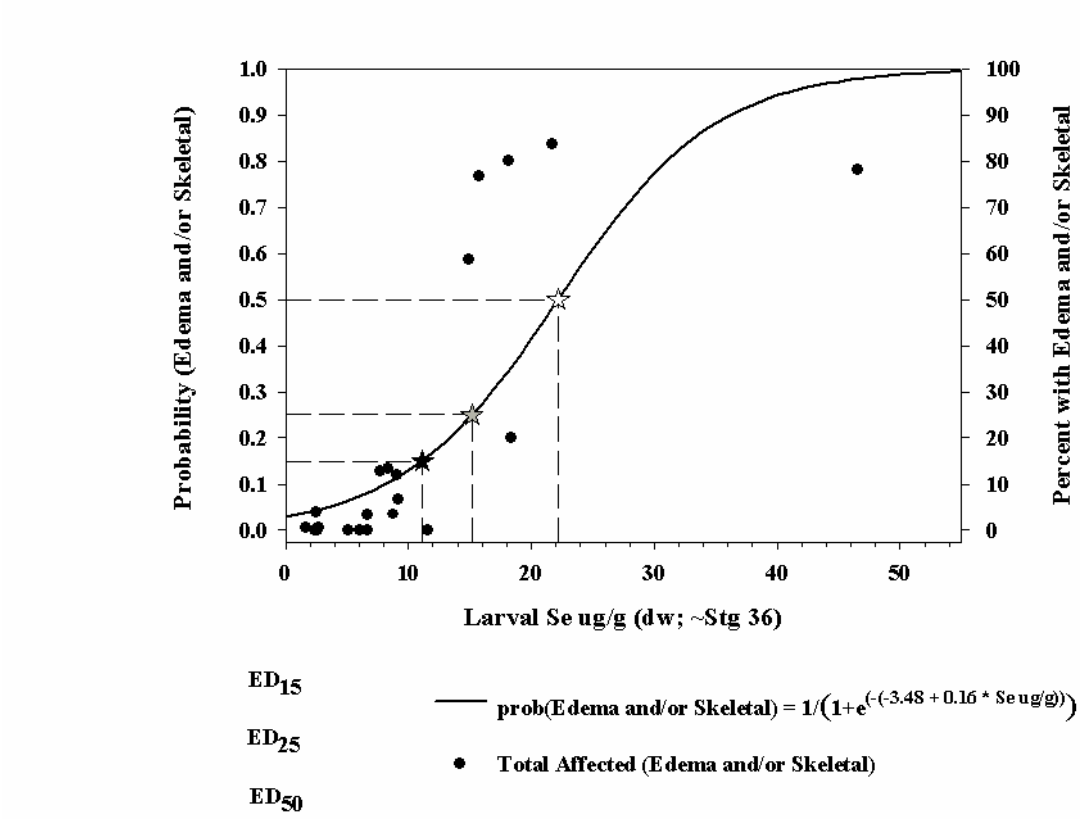
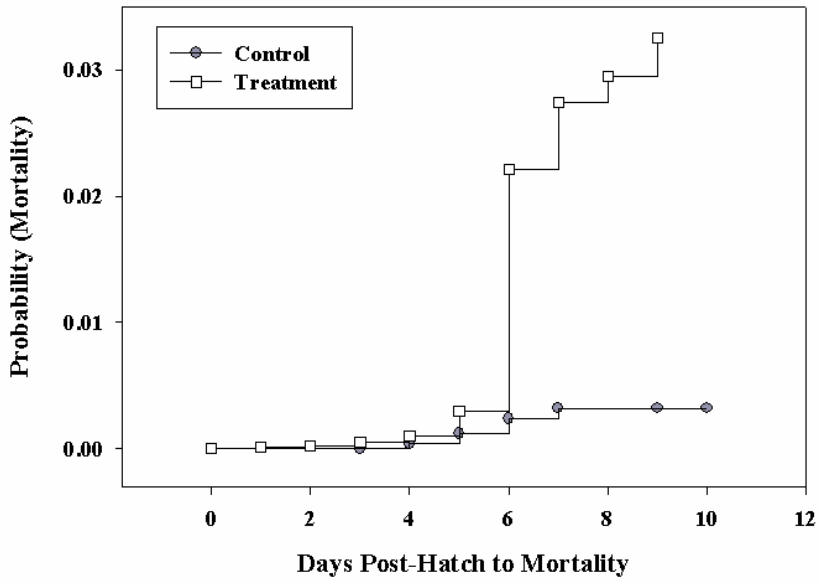
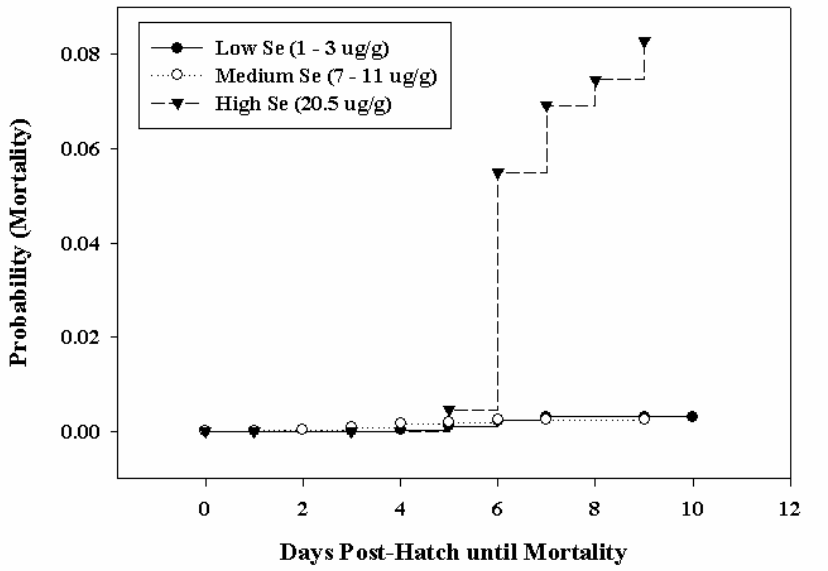


Figure 3-16. Percentage of larvae with edema and/or skeletal deformities related to selenium concentrations in larvae (μg/g; dw) at the start of larval development (*ca.* stage 36). Plot includes all data from the maternal exposure and microinjection experiments. Data includes nine progeny cohorts and spans early larval development (*ca.* stages 36 – 45). Logit analysis shows a significant association between selenium concentrations in larvae and the occurrence of defects ($\chi 2 = 543.2$, $p < 0.0001$; prob(*defects*) = $1/(1+e^{(-(-3.48 + 0.16 * (Se in Larvae ug/g))})$. The left y-axis shows the probability of larvae to develop edema and/or skeletal deformities at a given larval selenium concentration, as determined by logit analysis. The right y-axis shows the actual percentage of affected larvae in samples containing *ca.* 30 - 200 larvae each. ED_{50} (22.2 μg/g), ED_{25} (15.2 μg/g) and ED_{15} (11.1 μg/g) values were calculated from the logit equation.

Mortality in larvae from female white sturgeon exposed to dietary selenium

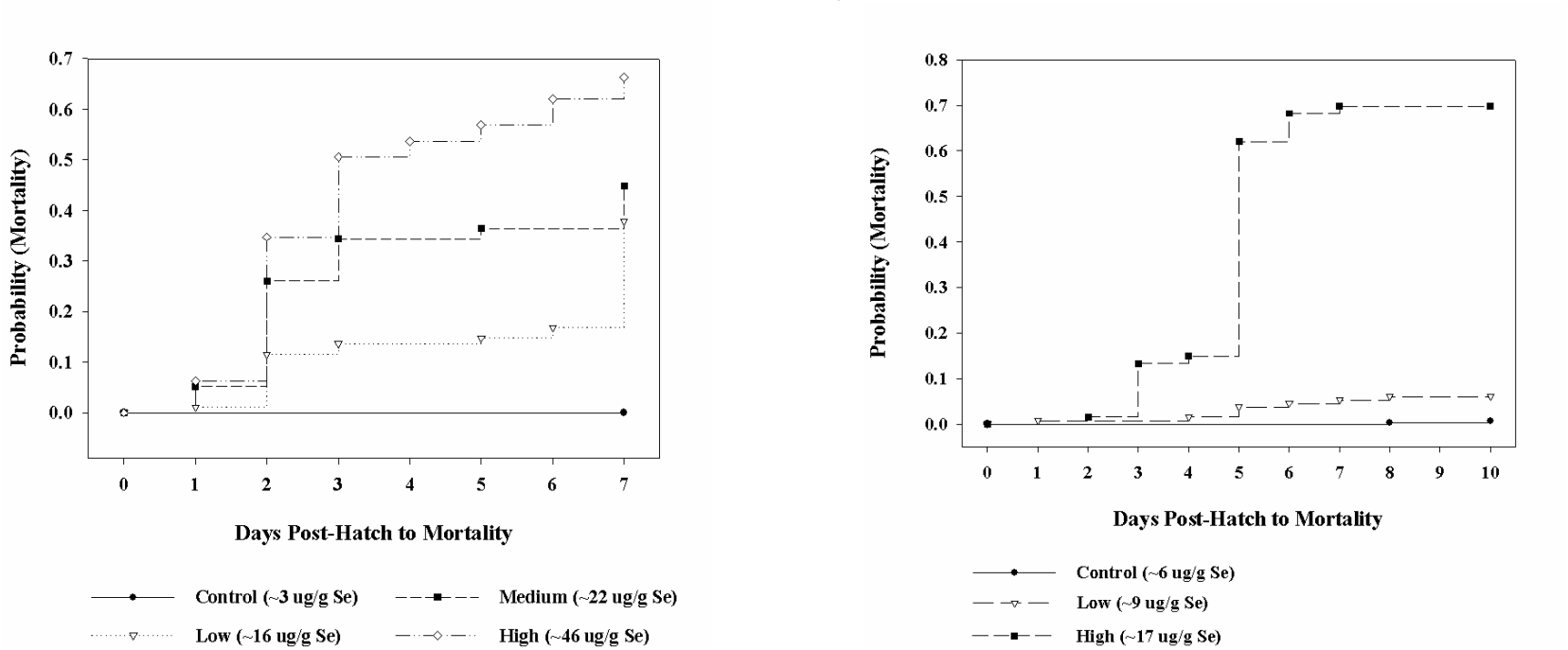


a)



b)

Figure 3-17. Probabilities of mortality in larvae from female white sturgeon exposed to dietary selenium. a) Kaplan-Meier analysis of time to larval mortality shows a statistically significant difference between control and treatment groups ($p < 0.0001$). b) When data are categorized by selenium concentrations in the eggs, a larger difference is detected in time to larval mortality (Kaplan-Meier; $p < 0.0001$). Differences between Kaplan-Meier distributions follow a) High >> Low > Medium, and b) Treatment > Control (Student's t-test, $p < 0.0001$).



a) Study One ***b) Study Two***

Figure 3-18. Probabilities of mortality in larvae following microinjection of seleno-L-methionine in ***a) Microinjection Study One*** and ***b) Microinjection Study Two***. Data are categorized by Se in larvae at the onset of larval development (*ca.* stage 36). In microinjection *study two*, the low and medium treatment groups were combined into one Se exposure group (low, *ca.* 9 µg/g Se; Table 3- 15). Control non-injected and sham-injected data were combined for both studies. Both studies show a statistically significant difference in the probability of larval mortality between exposure levels (Kaplan-Meier, $p < 0.0001$). Differences between Kaplan-Meier distributions follow a) High > Medium > Low > Control (Student's t-test, $p \leq 0.008$), and b) High > Low > Control (Student's t-test, $p < 0.001$).

Mortality in larvae from selenium maternal transfer and microinjection studies.

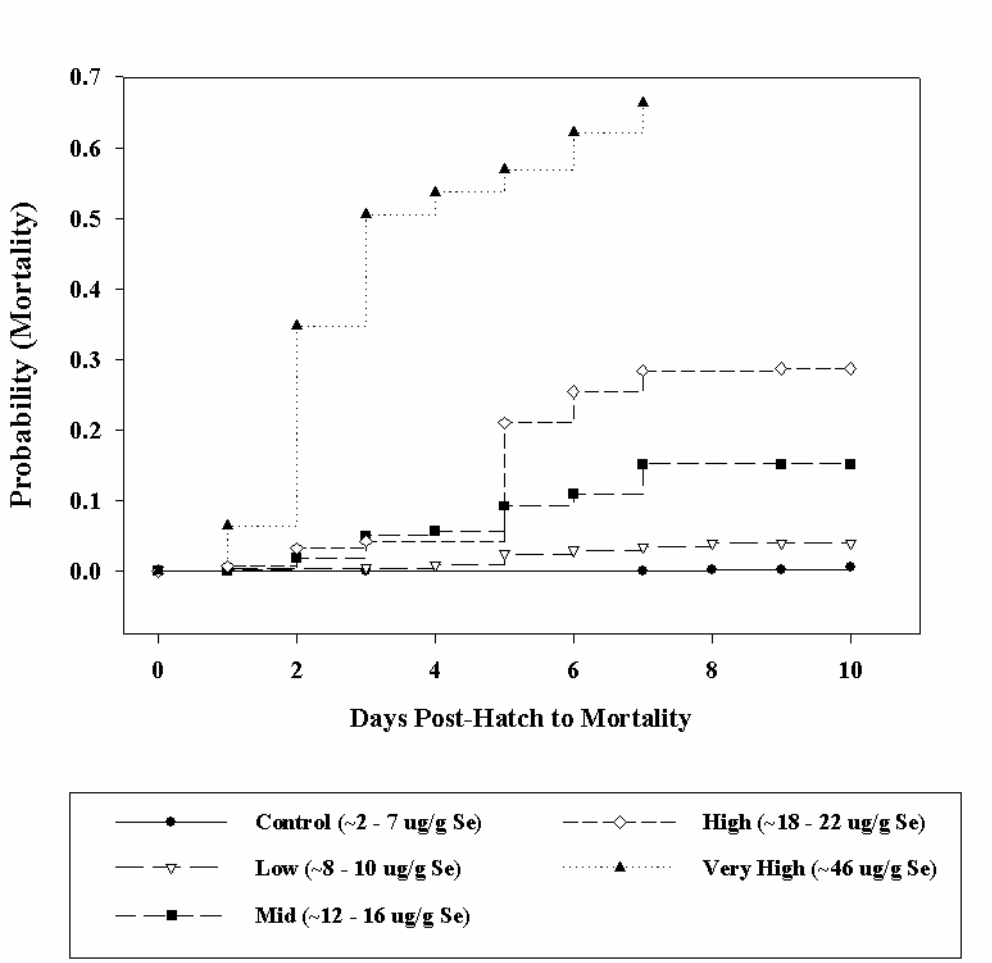


Figure 3-19. Probability of mortality in larvae using combined data from selenium maternal transfer and microinjection studies in white sturgeon. Kaplan-Meier analysis of time to larval mortality shows a statistically significant difference between exposure categories ($p < 0.0001$). Differences between Kaplan-Meier distributions follow Very High >> High = Mid > Low > Control (student's t-test, $p < 0.05$). Percent mortality presented in Table 3- 16.

Conclusions

This work shows that white sturgeon experience Se-induced toxicity at environmentally relevant dietary concentrations. Juveniles experienced cholestasis, a potentially fatal liver condition, when exposed to diets containing *ca.* 20 to 53 μg/g Se as selenized yeast (predominately selenomethionine). Adult females exposed to *ca.* 34 μg/g dietary Se transferred toxic levels of Se to their young through vitellogenesis. Our maternal transfer work is the first to experimentally demonstrate the incorporation of Se into the vitellogenin molecule, which supports the earlier hypothesis of Kroll and Doroshov (1991). Most importantly, we have shown that Se uptake into VTG, and ultimately the developing eggs, takes place in a dose-responsive manner. This indicates that even moderate increases, or decreases, in the accumulation of Se by reproductive females will affect the exposure level to their offspring. White sturgeon larvae containing Se concentrations above *ca.* 11 to 15 Se μg/g, following Se maternal transfer or microinjection, demonstrated significant increases of mortality and abnormality rates (including edema and skeletal deformities). Based on the work presented here, the hazard threshold for Se in developing white sturgeon should be set somewhere between 3 and 8 μg/g Se in yolk sac larvae (dw).

The microinjection of seleno-L-methionine into yolk sac larvae proved to be a reliable method to study the effects of excess Se on the development of this species. Microinjection was used to mimic maternal transfer because the large size and long ovarian cycle of white sturgeon make it difficult and expensive to study reproductive toxicity in this species under laboratory conditions. White sturgeon larvae tolerate

microinjection well, as indicated by absent or low mortality and defects in the sham-injected groups. This model may be useful in future studies.

The work presented here is novel because it exposed the actual species of ecological concern to the Se form (organic Se) and exposure route (dietary) that is predominant in the natural environment. Avoidance of using a surrogate species is especially important when studying sturgeon, since this phylogenetically ancient fish is significantly different from modern fish in anatomical and physiological characteristics. In addition, the narrow exposure range used here provides greater ecological significance by demonstrating the effects of relatively small increases in exposure.

In San Francisco Bay-Delta, juvenile white sturgeon are susceptible to toxicity at Se concentrations currently observed in some prey items (i.e., bivalves). In addition, juveniles in this region accumulate Se in their tissues at concentrations similar to those observed in our study. This leads us to suspect that juvenile white sturgeon in San Francisco Bay-Delta may be experiencing cholestasis, depending on individual dietary Se exposure. This potentially fatal liver condition disrupts the flow of bile, which prevents the excretion of many toxicants and can lead to direct toxicity by damaging liver tissue. The extent of cholestasis in juvenile white sturgeon from San Francisco Bay-Delta should be investigated. For adult white sturgeon in San Francisco Bay-Delta, typical Se exposure levels in the benthic food web (*ca.* 15 μg/g) are below the dietary Se level to which reproductive females were exposed in these experiments (*ca.* 34 μg/g). However, the experimental sturgeon accumulated Se concentrations in liver, muscle and eggs (*ca.* 10 – 12 μg/g Se) that are similar to body burdens of sturgeon in the San Francisco Bay population. The extent of accumulation in liver is most important here since we were

exploring the transport of accumulated Se from the liver to the developing eggs. Our research indicates that female white sturgeon in San Francisco Bay-Delta are likely transferring Se to their offspring. Limited data from this region supports this conclusion. Data from ten white sturgeon females sampled in the San Francisco Bay-Delta region show 3 to 29 μg/g Se (dw) in eggs or egg-bearing ovary (Kroll and Doroshov 1991; Doroshov Lab, UCD, unpublished data). Eight of those ten sturgeon exhibited egg Se levels above our proposed hazard threshold range of 3 to 8 μg/g Se in larvae (dw). Our maternal transport study showed strong conservation of Se between the egg and larval stages, indicating that egg Se concentrations may be interchangeable with larval concentrations (when both are calculated on a dry weight basis). Based on this information, it is likely that the accumulation of excess Se by female sturgeon in San Francisco Bay-Delta is affecting the recruitment of white sturgeon in this region. We observed severe edema and skeletal deformities in larvae containing Se concentrations above *ca.* 11 to 15 Se μg/g. These defects are expected to significantly decrease the probability of survival in white sturgeon larvae. The effected larvae have limited mobility and can be consumed by predators or may fail to start exogenous feeding. In this way, Se-induced developmental defects are an unseen factor in white sturgeon recruitment. White sturgeon eggs and gravid ovaries should be monitored for Se concentrations to determine the magnitude of potential impact on reproduction. Additionally, Se concentrations in the benthic food web should be routinely monitored since relatively small increases of Se in the food web can lead to increased toxicity to this species. Careful management of all processes with potential to increase Se

concentrations in the benthic food web is essential to protect sturgeon in San Francisco Bay-Delta and other high-Se systems.

References

Acton RT, Weinheimer PF, Dupree HK, Russell TR, Wolcott M, Evans ED, Schrohenloher RE, Bennett JC. 1971. Isolation and Characterization of the Immune Macro Globulin from the Paddlefish *Polyodon Spathula*. Journal of Biological Chemistry 246(22):6760-6769.

Adams WJ, Brix KV, Cothern KA, Tear LM, Cardwell RD, Fairbrother A, Toll JE. 1998. Assessment of selenium food chain transfer and critical exposure factors for avian wildlife species: Need for site-specific data. In: Little EE, DeLonay AJ, Greenberg BM, editors. Environmental Toxicology and Risk Assessment.

Adkison MA, Basurco B, Hedrick RP. 1996. Humoral immunoglobulins of the white sturgeon, *Acipenser transmontanus*: Partial characterization of and recognition with monoclonal antibodies. Developmental and Comparative Immunology 20(4):285-298.

Agius C. 1985. The melano-macrophage centres of fish. In: Manning MJaT, M.F., editor. Fish Immunology. London: Academic Press. p 85-105.

Akaishi FM, Silva de Assis HC, Jakobi SCG, Eiras-Stofella DR, St-Jean SD, Courtenay SC, Lima EF, Wagener ALR, Scofield AL, Ribeiro CAO. 2004. Morphological and neurotoxicological findings in tropical freshwater fish (*Astyanax sp.*) after waterborne and acute exposure to water soluble fraction (WSF) of crude oil. Archives of Environmental Contamination and Toxicology 46(2):244-253.

Anderson H, Moxon A. 1942. Changes in the blood picture of the dog following subcutaneous injections of sodium selenite. J Pharm. Exp. Ther. 76:343-354.

Anscombe FJ. 1948. The Transformation of Poisson, Binomial and Negative-Binomial Data. Biometrika 35(3/4):246-254.

Anundi I, Stahl A, Hogberg J. 1984. Effects of Selenite on 02 Consumption, Glutathione Oxidation and NADPH Levels in Isolated Hepatocytes and the Role of Redox Changes in Selenite Toxicity. Chemical-Biological Interactions 50:277-288.

Beer KE. 1981. Embryonic and larval development of white sturgeon (*Acipenser transmontanus*) [M.S.]: University of California, Davis. 93 p.

Beers MH, editor. 2003. The Merck Manual of Medical Information. 2nd ed. Whitehouse Station: Merck Research Laboratories.

Bennett W, Moyle P. 1996. Where have all the fishes gone: Interactive factors producing fish declines in the Sacramento-San Joaquin Estuary. In: Hollibaugh J, editor. San Francisco Bay The Ecosystem: Further investigations into the natural history of San Francisco Bay and Delta with reference to the influence of man. San Francisco: Pacific Division of American Association for the Advancement of Science.

Bernhart FW, Tomarelli RM. 1966. A salt mixture supplying the National Research Council estimates of the mineral requirements of the rat. Journal of Nutrition 89, 495-500.

Besser JM, Huckins JN, Clark RC. 1994. Separation of selenium species released from Se-exposed algae. Chemosphere 29(4):771-780.

Bidwell CA, Carlson DM. 1995. Characterization of vitellogenin from white sturgeon, *Acipenser transmontanus*. Journal of Molecular Evolution 41(1):104-112.

Billard R, Lecointre G. 2001. Biology and conservation of sturgeon and paddlefish. Reviews in Fish Biology and Fisheries 10(4):355-392.

Birstein V. 1993. Sturgeons and Paddlefishes: Threatened Fishes in Need of Conservation. Conservation Biology 7(4):773-787.

Black JJ, Maccubbin AE, Schiffert M. 1985. A Reliable Efficient Microinjection Apparatus and Methodology for the in-Vivo Exposure of Rainbow Trout *Salmo Gairdneri* and Salmon *Oncorhynchus Kisutch* Embryos to Chemical Carcinogens. Journal of the National Cancer Institute 75(6):1123-1128.

Blazer VS, Wolke RE, Brown J, Powell CA. 1987. Piscine Macrophage Aggregate Parameters as Health Monitors Effect of Age Sex Relative Weight Season and Site Quality in Largemouth Bass *Micropterus Salmoides*. Aquatic Toxicology (Amsterdam) 10(4):199-216.

Bolker JA. 1993. Gastrulation and mesoderm morphogenesis in the white sturgeon. Journal of Experimental Zoology 266(2):116-131.

Bonga S. 1997. The stress response in fish. Physiological Reviews 77(3):591-625.

Buddington RK, Doroshov SI. 1986. Structural and functional relations of the white sturgeon alimentary canal (*Acipenser transmontanus*). Journal of Morphology 190(2):201-214.

Burk RF, Hill KE, Read R, Bellew T. 1991. Response of Rat Selenoprotein P to Selenium Administration and Fate of Its Selenium. American Journal of Physiology 261(1 PART 1):E26-E30.

Byard JL. 1969. Trimethyl Selenide. A Urinary Metabolite of Selenite. Archives of Biochemistry and Biophysics 130(1-2):556.

Cai S-Y, Wang L, Ballatori N, Boyer JL. 2001. Bile salt export pump is highly conserved during vertebrate evolution and its expression is inhibited by PFIC type II mutations. American Journal of Physiology 281(2 Part 1):G316-G322.

California Department of Fish and Game Commission. 2006. Final statement of proposed emergency regulatory action. Emergency action to ammend sections 5.80, 27.60, 27.90 and 27.95, and add Sections 5.81 and 27.92, Title 14, California Code of Regulations. Changes to sturgeon bag and size limit.

Cappon CJ, Smith JC. 1982. Chemical form and distribution of mercury and selenium in edible seafood. J. Anal. Toxicol. 6:10-20.

Carlton JT, Thompson JK, Schemel LE, Nichols FH. 1990. Remarkable Invasion of San Francisco Bay California USA by the Asian Clam *Potamocorbula Amurensis* I. Introduction and Dispersal. Marine Ecology Progress Series 66(1-2):81-95.

Cleveland L, Little EE, Buckler DR, Wiedmeyer RH. 1993. Toxicity and bioaccumulation of waterborne and dietary selenium in juvenile bluegill (*Lepomis macrochirus*). Aquatic Toxicology (Amsterdam) 27(3-4):265-279.

Conte FS, Doroshov SI, Lutes PB, Strange EM. 1988. Hatchery manual for the White Sturgeon, *Acipenser transmontanus* Richardson, with application to other North American Acipenseridae. Oakland: Cooperative Extension, University of California, Division of Agriculture and Natural Resources.

Coughlan DJ, Velte JS. 1989. Dietary toxicity of selenium-contaminated red shiners to striped bass. Trans. Am. Fish. Soc. 118(4):400-408.

Coyle JJ, Buckler DR, Ingersoll CG, Fairchild JF, May TW. 1993. Effect of dietary selenium on the reproductive success of bluegill sunfish (*Lepomis macrochirus*). Environ. Toxicol. Chem. 12:551-565.

Crane M, Flower T, Holmes D, Watson S. 1992. The toxicity of selenium in experimental freshwater ponds. Arch. Environ. Contam. Toxicol. 23(4):440-452.

Deng X. 2000. Artificial reproduction and early life stages of the green sturgeon (*Acipenser medirostris*). [M.S.]: University of California, Davis. 62 p.

Dettlaff TA, Ginsburg AS, Shmalhausen OI. 1993. Sturgeon Fishes: Developmental Biology and Aquaculture. Berlin ; New York :: Springer-Verlag,. 300 p.

Diplock AT. 1976. Metabolic Aspects of Selenium Action and Toxicity. Critical Reviews in Toxicology 4(3):271-329.

Diplock AT, Caygill CPJ, Giasuddin ASM. 1976. Vitamin E Selenium Interactions. International Journal for Vitamin & Nutrition Research 46(2):254-256.

Doroshov S. 1985. Biology and Culture of Sturgeon Acipenseriformes. In: Muir J, Roberts R, editors. Recent Advances in Aquaculture. London & Sydney: Croom Helm. p 251-274.

Doroshov S, Moberg G, Van Eenennaam J. 1997. Observations on the reproductive cycle of cultured white sturgeon, *Acipenser transmontanus*. Environmental Biology of Fishes 48:265-278.

Doroshov SI. 2004. Observation of lipid-rich livers in cultured white sturgeon. Personal Communication.

Doroshov SI, Clark WH, Jr., Lutes PB, Swallow RL, Beer KE, McGuire AB, Cochran MD. 1983. Artificial propagation of the white sturgeon, *Acipenser transmontanus* Richardson. Aquaculture 35:117-123.

Drake EN. 2006. Cancer chemoprevention: Selenium as a prooxidant not an antioxidant. Medical Hypotheses 67(2):318-322.

Elferink RO. 2003. Cholestasis. Gut 52(Supplement 2):ii42-ii48.

Ellis MM, Motley HL, Ellis MD, Jones RO. 1937. Selenium poisoning in fishes. Proc. Soc. Exp. Biol. Med. 36:519-522.

Esaki N, Nakamura T, Tanaka H, Soda K. 1982. Selenocysteine Lyase, a Novel Enzyme That Specifically Acts on Selenocysteine - Mammalian Distribution and Purification and Properties of Pig-Liver Enzyme. Journal of Biological Chemistry 257(8):4386-4391.

Falchuk KH, Montorzi M. 2001. Zinc physiology and biochemistry in oocytes and embryos. BioMetals 14(3-4):385-395.

Fan TWM, Higashi RM, Lane AN. 1998. Biotransformation of selenium oxyanion by filamentous cyanophyte-dominated mat cultured from agricultural drainage waters. Environ. Sci. Technol. 32:3185-3193.

Fan TWM, Teh SJ, Hinton DE, Higashi RM. 2002. Selenium biotransformations into proteinaceous forms by foodweb organisms of selenium-laden drainage waters in California. Aquatic Toxicology (Amsterdam) 57(1-2):65-84.

Fletcher J, Copeland P, Driscoll D. 2000. Polysome distribution of phospholipids hydroperoxide glutathione peroxidase mRNA: evidence for a block in elongation at the UGA/selenocysteine codon. . RNA 6:1573–84.

Francesconi KA, Pannier F. 2004. Selenium metabolites in urine: A critical overview of past work and current status. Clinical Chemistry 50(12):2240-2253.

Frankenberger WT, Jr., Engberg RA. 1998. Environmental Chemistry of Selenium. New York: Marcel Dekker, Inc.

Garrett GP, Inmann CR. 1984. Selenium-induced changes in fish populations of a heated reservoir. Proc. Annu. Conf. Sourtheast. Assoc. Fish Wildlife Agencies 38:291-301.

Gatlin DM, III, Wilson RP. 1984a. Dietary selenium requirement of fingerling channel catfish. Journal of Nutrition 114(3):627-633.

Gatlin DM, III, Wilson RP. 1984b. Dietary selenium requirement of fingerling channel catfish. J. Nutr: 1984.

Georgiadis MP, Hedrick RP, Carpenter TE, Gardner IA. 2001. Factors influencing transmission, onset and severity of outbreaks due to white sturgeon iridovirus in a commercial hatchery. Aquaculture 194(1-2):21-35.

Ghosh P, Thomas P. 1995. Binding of metals to red drum vitellogenin and incorporation into oocytes. Marine Environmental Research 39(1-4):165-168.

Gillespie RB, Baumann PC. 1986. Effects of high tissue concentrations of selenium on reproduction by bluegills (*Lepomis macrochirus*). Transactions of the American Fisheries Society 115(2):208-213.

Gissel Nielsen M, Gissel Nielsen G. 1978. Sensitivity of trout to chronic and acute exposure to Selenium. Agriculture and Environment 4:85-91.

Gobler CJ, Hutchins DA, Fisher NS, Cosper EM, Sanudo-Wilhelmy SA. 1997. Release and Bioavailability of C, N, P, Se, and Fe Following Viral Lysis of a Marine Chrysophyte. Limnology and Oceanography 42(7):1492-1504.

Gromer S, Eubel J, Lee B, Jacob J. 2005. Human selenoproteins at a glance. Cell. Mol. Life Sci. 62:2414–2437.

Groothuis GMM, Meijer DKF. 1996. Drug traffic in the hepatobiliary system. Journal of Hepatology 24(SUPPL. 1):3-28.

Hamilton SJ. 1999. Hypothesis of historical effects from selenium on endangered fish in the Colorado River basin. Human & Ecological Risk Assessment 5(6):1153-1180.

Hamilton SJ. 2004. Review of selenium toxicity in the aquatic food chain. Science of the Total Environment 326(1-3):1-31.

Hamilton SJ, Buhl KJ. 1990. Acute Toxicity of Boron Molybdenum and Selenium to Fry of Chinook Salmon and Coho Salmon. Archives of Environmental Contamination & Toxicology 19(3):366-373.

Hamilton SJ, Buhl KJ, Faerber NL. 1990a. Toxicity of Organic Selenium in the Diet to Chinook Salmon. Environmental Toxicology and Chemistry 9(3):347-358.

Hamilton SJ, Buhl KJ, Faerber NL, Wiedmeyer RH, Bullard FA. 1990b. Toxicity of Organic Selenium in the Diet to Chinook Salmon. Environmental Toxicology & Chemistry 9(3):347-358.

Hamilton SJ, Holley KM, Buhl KJ. 2002a. Hazard assessment of selenium to endangered razorback suckers (*Xyrauchen texanus*). Science of the Total Environment 291(1-3):111-121.

Hamilton SJ, Holley KM, Buhl KJ, Bullard FA, Weston LK, McDonald SF. 2002b. Toxicity of selenium and other elements in food organisms to razorback sucker larvae. Aquatic Toxicology 59(3-4):253-281.

Hamilton SJ, Palmisano AN, Wedemeyer GA, Yasutake WT. 1986. Impacts of Selenium on Early Life Stages and Smoltification of Fall Chinook Salmon *Oncorhynchus Tshawytscha*. Transactions of the North American Wildlife & Natural Resources Conference:343-356.

Hampton JA, McCuskey PA, McCuskey RS, Hinton DE. 1985. Functional Units in Rainbow Trout *Salmo Gairdneri* Liver I. Arrangement and Histochemical Properties of Hepatocytes. Anatomical Record 213(2):166-175.

Heinz GH, Hoffman DJ, Gold LG. 1989. Impaired Reproduction of Mallards Fed an Organic Form of Selenium. Journal of Wildlife Management 53(2):418-428.

Helmy MH, Ismail SS, Fayed H, El-Bassiouni EA. 2000. Effect of selenium supplementation on the activities of glutathione metabolizing enzymes in human hepatoma Hep G2 cell line. Toxicology 144(1-3):57-61.

Hermanutz RO. 1992. Malformation of the Fathead Minnow *Pimephales Promelas* in an Ecosystem with Elevated Selenium Concentrations. Bulletin of Environmental Contamination & Toxicology 49(2):290-294.

Hermanutz RO, Allen KN, Roush TH, Hedtke SF. 1992. Effects of elevated selenium concentrations on bluegills (*Lepomis macrochirus*) in outdoor experimental streams. Environmental Toxicology & Chemistry 11(2):217-224.

Hicks BD, Hilton JW, Ferguson HW. 1984. Influence of Dietary Selenium on the Occurrence of Nephrocalcinosis in the Rainbow Trout *Salmo Gairdneri*. Journal of Fish Diseases 7(5):379-390.

Hill KE, Lloyd RS, Yang JG, Read R, Burk RF. 1991. The Complementary DNA for Rat Selenoprotein P Contains 10 Tga Codons in the Open Reading Frame. Journal of Biological Chemistry 266(16):10050-10053.

Hill KE, Zhou J, McMahan WJ, Motley AK, Atkins JF, Gesteland RF, Burk RF. 2003. Deletion of Selenoprotein P alters distribution of selenium in the mouse. Journal of Biological Chemistry 278(16):13640-13646.

Hilton JW, Hodson PV. 1983. Effect of increased dietary carbohydrate on selenium metabolism and toxicity in rainbow trout (*Salmo gairdneri*). Journal of Nutrition 113(6):1241-8.

Hilton JW, Hodson PV, Slinger SJ. 1980. The requirement and toxicity of selenium in rainbow trout (*Salmo gairdneri*). Journal of Nutrition 110(12):2527-35.

Hinton DE, Couch JA. 1998. Architectural pattern, tissue and cellular morphology in livers of fishes: Relationship to experimentally-induced neoplastic responses. Braunbeck, T., Hinton, D. E., Streit, B., editors; Fish Ecotoxicology. 1998. 141-164. Vol. 86.: Birkhaeuser Verlag, Basel; Birkhaeuser Boston, Inc., Massachusetts.

Hinton DE, Lantz RC, Hampton JA, McCuskey PR, McCuskey RS. 1987. Normal Versus Abnormal Structure Considerations in Morphologic Responses of Teleosts to Pollutants. Environmental Health Perspectives:139-146.

Hiramatsu N, Hiramatsu K, Hirano K, Hara A. 2002. Vitellogenin-derived yolk proteins in a hybrid sturgeon, bester (*Huso huso X Acipenser ruthenus*): Identification, characterization and course of proteolysis during embryogenesis. Comparative Biochemistry and Physiology Part A Molecular and Integrative Physiology 131A(2):429-441.

Hiramatsu N, Matsubara T, Fujita T, Sullivan CV, Hara A. 2006. Multiple piscine vitellogenins: biomarkers of fish exposure to estrogenic endocrine disruptors in aquatic environments. Marine Biology (Berlin) 149(1):35-47.

Hodson PV, Spry DJ, Blunt BR. 1980. Effects on rainbow trout (*Salmo gairdneri*)- of a chronic exposure to waterborne selenium. Can. J. Fish. Aquat. Sci. 37(2):233-240.

Hoffman DJ, Heinz GH. 1988. Embryotoxic and Teratogenic Effects of Selenium in the Diet of Mallards. Journal of Toxicology & Environmental Health 24(4):477-490.

Hoffman DJ, Heinz GH, Gold LG. 1988a. Embryotoxic and Teratogenic Effects of Inorganic and Organic Selenium in Mallards. Teratology 37(5):464.

Hoffman DJ, Heinz GH, Krynitsky AJ. 1989. Hepatic Glutathione Metabolism and Lipid Peroxidation in Response to Excess Dietary Selenomethionine and Selenite in Mallard Ducklings. Journal of Toxicology & Environmental Health 27(2):263-272.

Hoffman DJ, Heinz GH, Lecaptain LJ, Bunck CM, Green DE. 1991. Subchronic Hepatotoxicity of Selenomethionine Ingestion in Mallard Ducks. Journal of Toxicology & Environmental Health 32(4):449-464.

Hoffman DJ, Marn CM, Marois KC, Sproul E, Dunne M, Skorupa JP. 2002. Sublethal effects in avocet and stilt hatchlings from selenium-contaminated sites. Environmental Toxicology & Chemistry 21(3):561-566.

Hoffman DJ, Ohlendorf HM, Aldrich TW. 1988b. Selenium teratogenesis in natural populations of aquatic birds in central California. Arch. Environ. Contam. Toxicol. 17(4):519-525.

Hoffman JL. 1977. Selenite toxicity, depletion of liver S-adenosylmethionine, and inactivation of methionine adenosyltransferase. Archives of Biochemistry and Biophysics 179(1):136-40.

Holm J, Palace VP, Siwik P, Sterling G, Evans RE, Baron CL, Werner J, Wautier K. 2005. Developmental effects of bioaccumulated selenium in eggs and larvae of two salmonid speices. Environmental Toxicology and Chemistry 24(9):2373–2381.

Hu M, Yang Y, Martin JM, Yin K, Harrison PJ. 1997. Preferential uptake of Se(IV) over Se(VI) and the production of dissolved organic Se by marine phytoplankton. Marine Environmental Research 44(225-231).

Hung SSO. 1991. Sturgeon, *Acipenser spp.* In: Wilson RP, editor. Handbook of Nutrient Requirements of Finfish. Boca Raton: CRC Press, Inc. p 153-160.

Hung SSO. 2004. Observation of lipid-rich livers in cultured white sturgeon. Personal Communication.

Hung SSO, Deng DF. 2002. Sturgeon, *Acipenser spp.* In: Webster CD, Lim C, editors. Nutrient Requirements and Feeding of Finfish for Aquaculture. Wallingford, UK: CABI Publishing. p 344-357.

Hung SSO, Lutes PB. 1987. Optimum Feeding Rate of Hatchery-Produced Juvenile White Sturgeon *Acipenser Transmontanus* at 20 C. Aquaculture 65(3-4):307-318.

Hung SSO, Moore BJ, Bordner CE, Conte FS. 1987. Growth of juvenile white sturgeon (*Acipenser transmontanus*) fed different purified diets. Journal of Nutrition 117:328-334.

Jameson RR, Carlson BA, Butz M, Esser K, Hatfield DL, Diamond AM. 2002. Selenium influences the turnover of selenocysteine tRNA(Ser)Sec in Chinese hamster ovary cells. Journal of Nutrition 132(7):1830-1835.

Johns C, Luoma SN. 1988. Selenium Accumulation in Benthic Bivalves and Fine Sediments of San Francisco Bay, the Sacramento-San Joaquin Delta, and Selected Tributaries. Estuarine, Coastal and Shelf Science 27:381-396.

Kenedy-Stoskopf S. 1993. Immunology. In: Stoskopf MK, editor. Fish Medicine. Philadelphia: W.B. Saunders Company. p 149-159.

Kirby GM, Stalker MJ, Gordon S, Quinn BA, Van Schooten FJ, Hayes MA. 1995. Influences of chronic cholangiohepatitis and cholestasis on hepatic metabolism of benzo(a)pyrene in white suckers (*Catostomus commersoni*) from industrially polluted areas of Lake Ontario. Carcinogenesis 16(12):2923-2929.

Kobayashi Y, Ogra Y, Ishiwata K, Takayama H, Aimi N, Suzuki KT. 2002. Selenosugars are key and urinary metabolites for selenium excretion within the required to low-toxic range. Proceedings of the National Academy of Sciences of the United States of America 99(25):15932-15936.

Kohlhorst D. 1980. Recent trends in the White sturgeon population in California's Sacramento-San Joaquin Estuary. Calif Fish and Game 66(4):210-19.

Kohlhorst D, Miller L, Orsi J. 1980. Age and growth of White sturgeon collected in the Sacramento-San Joaquin Estuary, California: 1965-1970 and 1973-1976. Calif Fish and Game 66(2):83-95.

Kohlhorst DW, Botsford LW, Brennan JS, Cailliet GM. 1991. Aspects Of The Structure and Dynamics Of An Exploited Central California Population Of White Sturgeon (*Acipenser transmontanus*). In: Williot P, editor. Acipenser: Cemagref. p 277-293.

Kramer GF, Ames BN. 1988. Isolation and characterization of a selenium metabolism mutant of *Salmonella typhimurium*. J. Bacteriol. 170:736–743.

Kroll KJ. 1990. Preliminary characterization of vitellogenin and yolk proteins of the white sturgeon (*Acipenser transmontanus*). Davis: University of California. 85 p.

Kroll KJ, Doroshov SI. 1991. Vitellogenin: Potential Vehicle For Selenium Bioaccumulation In Oocytes Of White Sturgeon. In: Williot P, editor. Acipenser. Bordeaux: CEMAGREF. p 99-106.

Kryukov G, Gladyshev V. 2000. Selenium metabolism in zebrafish: multiplicity of selenoprotein genes and expression of a protein containing 17 selenocysteine residues. Genes to Cells 5:1049 -1060.

LaFleur GJ, Jr., Raldua D, Fabra M, Carnevali O, Denslow N, Wallace RA, Cerda J. 2005. Derivation of major yolk proteins from parental vitellogenins and alternative processing during oocyte maturation in *Fundulus heteroclitus*. Biology of Reproduction 73(4):815-824.

Lanfear J, Fleming J, Wu L, Webster G, Harrison PR. 1994. The selenium metabolite selenodiglutathione induces p53 and apoptosis: relevance to the chemopreventive effects of selenium? Carcinogenesis 15(7):1387-1392.

Lee R. 1993. Passage of xenobiotics and their metabolites from hepatopancreas into ovary and oocytes of blue crabs, *Callinectes sapidus*: Possible implications for vitellogenesis. Mar Environ Res. 35:181–187.

Lee R. 1996-2005. Diagnostic Liver Pathology: Electronic version by R. Bourne/University of Pittsburgh Medical Center.

Lemly AD. 1982. Response of juvenile centrachids to sublethal concentrations of waterborne selenium. I. Uptake, tissue distribution, and retention. Aquatic Toxicology 2:235-252.

Lemly AD. 1985. Toxicology of selenium in a freshwater reservoir: Implications for environmental hazard evaluation and safety. Ecotoxicol. Environ. Saf. vol. 10(no. 3):pp. 314-338.

Lemly AD. 1993a. Metabolic stress during winter increases the toxicity of selenium to fish. Aquatic Toxicology (Amsterdam) 27(1-2):133-158.

Lemly AD. 1993b. Teratogenic effects of selenium in natural populations of freshwater fish. Ecotoxicology and Environmental Safety 26(2):181-204.

Lemly AD. 1998. Pathology of selenium poisoning in fish. In: Frankenberger WTJ, Engberg RA, editors. Environmental chemistry of selenium.: Marcel Dekker, Inc. p 281-296.

Lemly AD. 2002. Selenium assessment in aquatic ecosystems: A guide for hazard evaluation and water quality criteria. New York: Springer-Verlag New York Inc.

Linares-Casenave J, Van Eenennaam JP, Doroshov SI. 2002. Ultrastructural and histological observations on temperature-induced follicullar ovarian atresia in the white sturgeon. Journal of Applied Ichthyology 18(4-6):382-390.

Linares J, Linville RG, Van Eenennaam JP, Doroshov SI. 2004. Selenium Effects on Health and Reproduction of White Sturgeon in the Sacramento-San Joaquin Estuary. Davis: University of California. Report nr Final Report for CALFED Project No. ERP-02-P35

Linville RG, Luoma SN, Cutter L, Cutter GA. 2002. Increased selenium threat as a result of invasion of the exotic bivalve *Potamocorbula amurensis* into the San Francisco Bay-Delta. Aquatic Toxicology (Amsterdam) 57(1-2):51-64.

Lorent K, Yeo S-b, Oda T, Chandrasekharappa S, Chitnis A, Matthews RP, Pack M. 2004. Inhibition of Jagged-mediated Notch signaling disrupts zebrafish biliary development and generates multi-organ defects compatible with an Alagille syndrome phenocopy. Development 131(22):5753-5766.

Luoma S, Linville R. 1997. Selenium trends in North San Francisco Bay. Interagency Ecological Program for the Sacramento-San Joaquin Estuary Newsletter 10:25-26.

Luoma SN, Johns C, Fisher NS, Steinberg NA, Oremland RS, Reinfelder JR. 1992. Determination of selenium bioavailability to a benthic bivalve from particulate and solute pathways. Environ. Sci. & Tech. 26(3):485-491.

Luoma SN, Presser TS. 2000. Forecasting Selenium Discharges to the San Francisco Bay-Delta Estuary: Ecological Effects of a Proposed San Luis Drain Extension. Menlo Park: United States Geological Survey. Report nr 00-416. 157 p.

Martin JL. 1973. Selenium assimilation in animals. In: Klayman DL, Gunther WHH, editors. Organic Selenium Compounds: Their Chemistry and Biology. p 663-691.

Mayland H. 1994. Selenium in plant and animal nutrition. In: Frankenberger WTJ, Benson S, editors. Selenium in the Environment. New York: Marcel Dekker, Inc. p 29-45.

McCabe GT, Jr., Emmett RL, Hinton SA. 1993. Feeding ecology of juvenile white sturgeon (*Acipenser transmontanus*) in the lower Columbia River. Northwest Science 67(3):170-180.

McConnell K, Portman O. 1952. Toxicity of dimethylselenide in the rat and mouse. Proc Soc Exp Biol Med 79:230 - 231.

McKechnie RJ, Fenner RB. 1971. Food Habits of White Sturgeon *Acipenser Transmontanus* in San-Pablo and Suisun Bays California. California Fish and Game 57(3):209-212.

Metcalfe CD, Cairns VW, Fitzsimons JD. 1988. Microinjection of Rainbow Trout at the Sac-Fry Stage a Modified Trout Carcinogenesis Assay. Aquatic Toxicology (Amsterdam) 13(4):347-356.

Miller WT, Schoening HW. 1938. Toxicity of selenium fed to swine in the form of sodium selenite. J. Agr. Res. 56:831.

Mims SD, Lazur A, Shelton WL, Gomelsky B, Chapman F. 2002. Species profile. Production of Sturgeon. Southern Regional Aquaculture Center. Publ. No. 7200.

Mommsen TP, Walsh PJ. 1988. Vitellogenesis and oocyte assembly. In: W.S. H, .D.J. R, editors. The physiology of developing fish. San Diego: Academic Press.

Monteverdi GH, Di Giulio RT. 2000. In vitro and in vivo association of 2,3,7,8-tetrachlorodibenzo-p-dioxin and benzo(a)pyrene with the yolk-precursor protein vitellogenin. Environmental Toxicology & Chemistry 19(10):2502-2511.

Mostert V. 2000. Selenoprotein P: properties, functions and regulation. . Arch. Biochem. Biophys. 376: :433–438.

Motchnik PA, Tappel AL. 1990. Multiple Selenocysteine Content of Selenoprotein P in Rats. Journal of Inorganic Biochemistry 40(3):265-270.

Müller S, Senn H, Gsell B, Vetter W, Baron C, Böck A. 1994. The formation of diselenide bridges in proteins by incorporation of selenocysteine residues: biosynthesis and characterization of (Se)2-thioredoxin. Biochemistry 33:3404–3412.

Nathanson MH, Boyer JL. 1991. Mechanisms and Regulation of Bile Secretion. Hepatology 14(3):551-566.

Neter J, Kutner M, Wasserman W, Nachtsheim C, Neter J. 1996. Applied linear regression models. Chicago: Irwin. 720 p.

Nichols FH, Thompson JK, Schemel LE. 1990. Remarkable Invasion of San Francisco Bay California USA by the Asian Clam *Potamocorbula Amurensis* II. Displacement of a Former Community. Marine Ecology Progress Series 66(1-2):95-102.

O'Toole D, Raisbeck M. 1997. Experimentally Induced Selenosis of Adult Mallard Ducks: Clinical Signs, Lesions, and Toxicology. Vet. Pathol. 34:330-340.

Ohlendorf HM, Hoffman DJ, Saiki MK, Aldrich TW. 1986. Embryonic Mortality and Abnormalities of Aquatic Birds Apparent Impacts of Selenium from Irrigation Drainwater. Science of the Total Environment 52(1-2):49-64.

Ohlendorf HM, Kilness AW, Simmons JL, Stroud RK, Hoffman DJ, Moore JF. 1988. Selenium Toxicosis in Wild Aquatic Birds. Journal of Toxicology & Environmental Health 24(1):67-92.

Okuno T, Motobayashi S, Ueno H, Nakamuro K. 2005a. Identification of mouse selenomethionine alpha,gamma-elimination enzyme - Cystathionine gamma-lyase catalyzes its reaction to generate methylselenol. Biological Trace Element Research 108(1-3):245-257.

Okuno T, Motobayashi S, Ueno H, Nakamuro K. 2005b. Purification and characterization of mouse hepatic enzyme that converts selenomethionine to methylselenol by its alpha,gamma-elimination. Biological Trace Element Research 106(1):77-93.

Oremland RS, Steinberg NA, Maest AS, Miller LG, Hollibaugh JT. 1990. Measurement of in situ rates of selenate removal by dissimilatory bacterial reduction in sediments. Environmental Science & Technology 24(8):1157-1164.

Palace VP, Spallholz JE, Holm J, Wautier K, Evans RE, Baron CL. 2004. Metabolism of selenomethionine by rainbow trout (*Oncorhynchus mykiss*) embryos can generate oxidative stress. Ecotoxicology & Environmental Safety 58(1):17-21.

Palmeira CM, Rolo AP. 2004. Mitochondrially-mediated toxicity of bile acids. Toxicology 203(1-3):1-15.

Peri S. *et al.* 2003. Development of human protein reference database as an initial platform for approaching systems biology in humans. . Genome Research. 13 2363-2371.

Plack PA, Skinner ER, Rogie A, Mitchell AI. 1979. Distribution of Ddt between the Lipo Proteins of Trout Serum. Comparative Biochemistry and Physiology C Comparative Pharmacology 62(1):119-126.

Polatajko A, Banas B, Encinar JR, Szpunar J. 2005. Investigation of the recovery of selenomethionine from selenized yeast by two-dimensional LC-ICP MS. Analytical and Bioanalytical Chemistry 381(4):844-849.

Presser TS, Ohlendorf HM. 1987. Biogeochemical Cycling of Selenium in the San Joaquin Valley California USA. Environmental Management 11(6):804-822.

Quinlan GJ, Martin GS, Evans TW. 2005. Albumin: Biochemical properties and therapeutic potential. Hepatology 41(6):1211-1219.

Radtke I. 1966. Distribution of smelt, juvenile sturgeon, and starry flounder in the Sacramento-San Joaquin Delta with observations on food of sturgeon. In: Turner J, Delley D, editors. Ecological studies of the Sacramento-San Joaquin Delta. Part II, Fishes of the Delta.: California Department of Fish and Game. p 115-129.

Reddy CC, Massaro EJ. 1983. Biochemistry of selenium: a brief overview. Fundamental and Applied Toxicology 3(5):431-6.

Reinfelder JR, Wang WX, Luoma SN, Fisher NS. 1997. Assimilation efficiencies and turnover rates of trace elements in marine bivalves: A comparison of oysters, clams and mussels. Marine Biology (Berlin) 129(3):443-452.

Rice C. 2001. Fish immunotoxicology: Understanding mechanisms of action. In: Benson DSaWH, editor. Target Organ Toxicity in Marine and Freshwater Teleosts. London: Taylor and Francis. p 96-138.

Risebrough RW, Chapman JW, Okazaki rK, Schmidt TT. 1977. Toxicants in San Francisco Bay and Estuary. Berkeley, CA: Association of Bay Area Governments.

Rolo AP, Palmeira CM, Holy JM, Wallace KB. 2004. Role of mitochondrial dysfunction in combined bile acid-induced cytotoxicity: The switch between apoptosis and necrosis. Toxicological Sciences 79(1):196-204.

Romano M, Rosanova P, Anteo C, Limatola E. 2004. Vertebrate yolk proteins: A review. Molecular Reproduction and Development 69(1):109-116.

Saito Y, Takahashi K. 2002. Characterization of selenoprotein P as a selenium supply protein. European Journal of Biochemistry 269(22):5746-5751.

Schaffter RG, Kohlhorst DW. 1999. Status of white sturgeon in the Sacramento-San Joaquin Estuary. California Fish & Game 85(1):37-41.

Schmalhausen OI. 1991. The Russian sturgeon *Acipenser guldenstadti* . Part II. Later prelarval development. In: Dettlaff TA, Vassetzky SG, editors. Animal Species for Developmental Studies. New York: Consultants Bureau.

Schmitt M, Kubitz R, Wettstein M, vom Dahl S, Haeussinger D. 2000. Retrieval of the Mrp2 gene encoded conjugate export pump from the canalicular membrane contributes to cholestasis induced by tert-butyl hydroperoxide and chloro-dinitrobenzene. Biological Chemistry 381(5-6):487-495.

Schrauzer GN. 2000. Selenomethionine: A review of its nutritional significance, metabolism and toxicity. Journal of Nutrition 130(7):1653-1656.

Schreiber M. 1962. Observations on the food habits of juvenile white sturgeon. California Fish and Game 48(1):79-80.

Schultz R, Hermanutz R. 1990. Transfer of Toxic Concentrations of Selenium from Parent to Progeny in the Fathead Minnow *Pimephales Promelas*. Bulletin of Environmental Contamination & Toxicology 45(4):568-573.

Schweizer U, Streckfuss F, Pelt P, Carlson BA, Hatfield DL, Koehrle J, Schomburg L. 2005. Hepatically derived selenoprotein P is a key factor for kidney but not for brain selenium supply. Biochemical Journal 386:221-226.

Seko Y, Saito Y, Kitahara J, Imura N. Active Oxygen Generation by the Reaction of Selenite with Reduced Glutathione in-Vitro. In: Wendel A, editor; 1989; Tuebingen, West Germany. Springer-Verlag New York, Inc. p 70-73.

Sewertzoff AN. 1923. The place of the cartilaginous Ganoids in the system and the evolution of the Osteichthyes. Journal of Morphology 38(1):105-145.

Shackley SE, King PE, Gordon SM. 1981. Vitellogenesis and Trace Metals in a Marine Teleost *Blennius Pholis*. Journal of Fish Biology 18(3):349-352.

Shiobara Y, Suzuki KT. 1998. Binding of selenium (administered as selenite) to albumin after efflux from red blood cells. Journal of Chromatography B 710(1-2):49-56.

Singh R, Randhawa SS, Dhillon KS. 2002. Changes in blood biochemical and enzyme profile in experimental chronic selenosis in buffalo calves (*Bubalus bubalis*). Indian Journal of Animal Sciences 72(3):230-232.

Skorupa JP. 1998. Selenium Poisoning of Fish and Wildlife in Nature: Lessons from Twelve Real-World Examples. In: Frankenberger WT, Jr., Engberg RA, editors. Environmental Chemistry of Selenium. New York: Marcel Dekker, Inc. p 315-354.

Sokol RJ, Winklhofer-Roob BM, Devereaux MW, McKim JM, Jr. 1995. Generation of Hydroperoxides in Isolated Rat Hepatocytes and Hepatic Mitochondria Exposed to Hydrophobic Bile Acids. Gastroenterology 109(4):1249-1256.

Sorensen E. 1988. Selenium accumulation, reproductive status, and histopathological changes in environmentally exposed redear sunfish. Arch Toxicol 61(4):324 - 329.

Sorensen E, Thomas P. 1988. Selenium accumulation, reproductive status, and histopathological changes in environmentally exposed redear sunfish. Arch Toxicol 61(4):324-9.

Sorensen EM, Bauer TL. 1984. A correlation between selenium accumulation in sunfish and changes in condition factor and organ weight. Environ. Pollut. 34:357-366.

Sorensen EM, Bauer TL, Bell JS, Harlan CW. 1982a. Selenium accumulation and cytotoxicity in teleosts following chronic, environmental exposure. Bulletin of Environmental Contamination and Toxicology 29(6):688-96.

Sorensen EM, Bell JS, Harlan CW. 1983a. Histopathological changes in selenium-exposed fish. American Journal of Forensic Medicine and Pathology 4(2):111-23.

Sorensen EM, Harlan CW, Bell JS. 1982b. Renal changes in selenium-exposed fish. American Journal of Forensic Medicine and Pathology 3(2):123-9.

Sorensen EM, Harlan CW, Bell JS, Bauer TL, Pradzynski AH. 1983b. Hepatocyte changes following selenium accumulation in a freshwater teleost. American Journal of Forensic Medicine and Pathology 4(1):25-32.

Sorensen EMB. 1986. The Effects of Selenium on Freshwater Teleosts. In: Hodgson E, editor. .Reviews in Environmental Toxicology, 2. Amsterdam: Elsevier Science Publishers B.V. p 59-116.

Sorensen EMB, Cumbie PM, Bauer TL, Bell JS, Harlan CW. 1984. Histopathological, hematological, condition-factor, and organ weight changes associated with selenium accumulation in fish from Belews Lake, North Carolina. Archives of Environmental Contamination & Toxicology 13(2):153-162.

Spallholz JE. 1994. On the Nature of Selenium Toxicity and Carcinostatic Activity. Free Radical Biology and Medicine 17(1):45-64.

Spallholz JE, Hoffman DJ. 2002. Selenium toxicity: Cause and effects in aquatic birds. Aquatic Toxicology (Amsterdam) 57(1-2):27-37.

Spallholz JE, Palace VP, Reid TW. 2004. Methioninase and selenomethionine but not Se-methylselenocysteine generate methylselenol and superoxide in an in vitro chemiluminescent assay: Implications for the nutritional carcinostatic activity of selenoamino acids. Biochemical Pharmacology 67(3):547-554.

Stadtman TC. 1974. Selenium biochemistry. Science 183(128):915-22.

Stanley TR, Smith GJ, Hoffman DJ, Heinz GH, Rosscoe R. 1996. Effects of Boron and Selenium On Mallard Reproduction and Duckling Growth and Survival. Environmental Toxicology and Chemistry 15(7):1124-1132.

Stewart AR, Luoma SN, Schlekat CE, Doblin MA, Hieb KA. 2004. Food web pathway determines how selenium affects aquatic ecosystems: A San Francisco Bay case study. Environmental Science & Technology 38(17):4519-4526.

Suzuki K. 2005a. Metabolomics of selenium: Se metabolites based on speciation studies. Journal of Health Science 51(2):107-114.

Suzuki KT. 2005b. Metabolomics of selenium: Se metabolites based on speciation studies. Journal of Health Science 51(2):107-114.

Tallandini L, Cecchi R, Deboni S, Galassini S, Ghermandi G, Gialanella G, Liu NQ, Moro R, Turchetto M, Zhang YX. 1996. Toxic Levels of Selenium in Enzymes and Selenium Uptake in Tissues of a Marine Fish. Biological Trace Element Research 51(1):97-106.

Tashjian D, Teh S, Sogomonyan A, Hung S. 2006. Bioaccumulation and chronic toxicity of dietary l-selenomethionine in juvenile white sturgeon (*Acipenser transmontanus*). Aquatic Toxicology 79:401–409.

Tashjian DH. 2005. Selenium toxicokinetics, chronic toxicity, and interaction with salinity stress in white sturgeon [Ph.D.]. United States -- California: University of California, Davis.

Teh SJ, Deng X, Deng D-F, Teh F-c, Hung SSO, Fan TWM, Liu J, Higashi RM. 2004. Chronic effects of dietary selenium on juvenile Sacramento splittail (*Pogonichthys macrolepidotus*). Environmental Science & Technology 38(22):6085-6093.

Thissea C, Degravea A, Kryukovb G, Gladyshevb V, Obrecht-Pflumioa S, Krolc A, Thissea B, Lescurec A. 2003. Spatial and temporal expression patterns of selenoprotein genes during embryogenesis in zebrafish. Gene Expression Patterns 3:525–532.

Trauner M, Meier PJ, Boyer JL. 1998. Molecular pathogenesis of cholestasis. New England Journal of Medicine 339(17):1217-1227.

Trauner M WM, Fickert P, Zollner G. 2005. Molecular regulation of hepatobiliary transport systems: clinical implications for understanding and treating cholestasis. J Clin Gastroenterol. 39(4 Suppl 2):S111-24.

Tujebajeva R, Ransom D, Harney J, Berry M. 2000. Expression and characterization of nonmammalian selenoprotein P in the zebrafish, *Danio rerio*. Genes to Cells 5:897-903.

Ungerer JR, Thomas P. 1996. Role of very low density lipoproteins in the accumulation of o,p'-DDT in fish ovaries during gonadal recrudescence. Aquatic Toxicology (Amsterdam) 35(3-4):183-195.

Urquhart KAF, Regalado K. 1991. Selenium Verification Study 1988 - 1990. Sacramento: California Department of Fish and Game. Report nr 91-2-WQ. 95 p.

USACE. 2003. Final biological assessment on the operation of the Missouri river mainstem reservoir system, the operation and maintenance of the bank stabilization and navigation project, and the operation of Kansas river reservoir system.: US Army Corps of Engineers. 30 p.

Van Eenennaam JP, Chapman FA, Jarvis PL. 2004. Aquaculture. Fish and Fisheries Series. p 277-311.

Vandermeulen JH, Foda A. 1988. Cycling of selenite and selenate in marine phytoplankton. Marine Biology 98:115-123.

Velinsky DJ, Cutter GA. 1991. Geochemistry of Selenium in a Coastal Salt Marsh. Geochimica et Cosmochimica ACTA 55(1):179-191.

Vernie LN, Bont WS, Emmelot P. 1974. Inhibition of in vitro amino acid incorporation by sodium selenite. Biochemistry 13(2):337-41.

Vernie LN, Van Leewenhoekhuis A. 1987. Inhibition of Protein Synthesis and Anticarcinogenicity of Selenium Compounds. p 1074-1083.

Vethaak AD, Jol JG, Meijboom A, Eggens ML, Ap Rheinallt T, Wester PW, Van De Zande T, Bergman A, Dankers N, Ariese F and others. 1996. Skin and liver diseases induced in flounder (*Platichthys flesus*) after long-term exposure to contaminated sediments in large-scale mesocosms. Environmental Health Perspectives 104(11):1218-1229.

Vethaak AD, Wester PW. 1996. Diseases of flounder Platichthys flesus in Dutch coastal and estuarine waters, with particular reference to environmental stress factors. II. Liver histopathology. Diseases of Aquatic Organisms 26(2):99-116.

Wallace RA, Morgan JP. 1986. Isolation of Phosvitin Retention of Small Molecular Weight Species and Staining Characteristics on Electrophoretic Gels. Analytical Biochemistry 157(2):256-261.

Wang D, Lovell RT. 1997. Organic selenium sources, selenomethionine and selenoyeast, have higher bioavailability than an inorganic selenium source, sodiuim selenite, in diets for channel catfish (*Ictalurus punctatus*). Aquaculture 152:223-234.

Wang WX, Guo L. 2001. Production of Colloidal Organic Carbon and Trace Metals by Phytoplankton Decomposition. Limnology and Oceanography 46(2):278-286.

Wang YL, Binkowski FP, Doroshov SI. 1985. Effect of temperature on early development of white and lake sturgeon, *Acipenser transmontanus* and *Acipenser fulvescens*. In: Binkowski FP, Doroshov SI, editors. North American Sturgeons: biology and aquaculture potential. Dordercht: Dr. W. Jung Publishers. p 43-50.

Wester PW, Vethaak AD, Van Muiswinkel WB. 1994. Fish as biomarkers in immunotoxicology. Toxicology 86(3):213-232.

White JR, Hofmann PS, Hammond D, Baumgartner S. 1987. Selenium Verification Study 1986. California Department of Fish and Game.

White JR, Hofmann PS, Hammond D, Baumgartner S. 1988. Selenium Verification Study 1986-1987. California Department of Fish and Game.

White JR, Hofmann PS, Urquhart KAF, Hammond D, Baumgartner S. 1989. Selenium Verification Study 1987-1988. California Department of Fish and Game.

Wolke RE, Murchelano RA, Dickstein CD, George CJ. 1985. Preliminary Evaluation of the Use of Macrophage Aggregates as Fish Health Monitors. Bulletin of Environmental Contamination and Toxicology 35(2):222-227.

Woock S, Garrett W, Partin W, Bryson W. 1987. Decreased survival and teratogenesis during laboratory selenium exposures to bluegill, *Lepomis macrochirus*. Bull Environ Contam Toxicol 39(6):998-1005.

Wrench JJ. 1978. Selenium Metabolism in the Marine Phytoplankters *Tetraselmis tetrathele* and *Dunaliella minuta*. Marine Biology 49:231-263.

Wrobel KH. 2003. The genus Acipenser as a model for vertebrate urogenital development: the mullerian duct. Anatomy and Embryology 206:255-271.

Wu L, Lanfear J, Harrison PR. 1995. The Selenium Metabolite Selenodiglutathione Induces Cell-Death by a Mechanism Distinct from H2O2 Toxicity. Carcinogenesis 16(7):1579-1584.

Yan L, Spallholz JE. 1991. Free Radical Generation by Selenium Compounds. FASEB Journal 5(4):A581.

Zar JH. 1999. Biostatistical analysis. Upper Saddle River, New Jersey: Prentice Hall. 663 p.

Appendix A. Biliary transport and cholestasis in fish

Hepato-biliary transport of biliary constituents and their precursors from blood to bile includes three major steps. First, the uptake of substances from blood plasma across the sinusoidal membrane and into the hepatocytes must occur. This is followed by intracellular transport and metabolism. Finally, the transport and excretion of the parent substance or metabolites across hepatocyte membranes into biliary passageways occurs (Groothuis and Meijer 1996; Nathanson and Boyer 1991). Bile flow is primarily controlled by the energy dependent (ATP-dependent) secretion of biliary constituents into bile canaliculi and the resulting osmotic flow of water (Elferink 2003).

In the well-studied mammalian model, bile salts and organic ions are removed from sinusoidal blood by Na^+-dependent Na^+/taurocholate cotransporters (NTCP) and Na^+-independent organic anion transporting proteins (OATP) in hepatocyte basolateral membranes (Ferenci *et al.* 2002; Trauner and Boyer 2003). Following metabolism and intracellular transport, bile constituents are secreted from several different transport proteins in hepatocyte canalicular membranes, including both adenosine triphosphate (ATP)-dependent and –independent transport systems. The ATP-dependent transport proteins include multi-drug resistance proteins (MDR), multidrug-associated proteins (MRP) and bile salt export pumps (BSEP), which are related to P-glycoprotein (Arrese and Ananthanarayanan 2004; Trauner and Boyer 2003). These ATP-dependent transport proteins remove bile salts, phospholipids, cholesterol, organic ions, phase II conjugates, toxins, and other bile constituents from hepatocytes into biliary canaliculi (Arrese and Trauner 2003; Ferenci *et al.* 2002; Trauner and Boyer 2003). ATP-independent chloride-bicarbonate anion exchanger protein (AE2), found in hepatocyte and bile duct epithelial

cell membranes, secretes bicarbonate into the bile flow (Kanno *et al.* 2001; Trauner and Boyer 2003). Bile collected in canaliculi, which are formed by the spaces between hepatocytes, flows into intrahepatic bile ductules and ducts, which are formed by biliary epithelial cells. Bile exits the liver through the hepatic bile ducts and is transported to the gall bladder and the intestinal lumen by the larger cystic and common bile ducts.

The architecture of fish liver differs significantly from that of mammals (Hinton and Couch 1998). Teleosts livers exhibit tubular architecture, characteristic of the lower vertebrates, with canaliculi formed by basolateral plasma membranes of adjacent hepatocytes and transitional biliary passageways, the tubular lumena, lined by apical plasma membrane of hepatocytes and by transitional biliary epithelial cells, the bile preductular epithelial cells (Hampton *et al.* 1988). White sturgeon also has a tubular liver, as demonstrated in the first chapter of this dissertation. Biliary transport is only beginning to be understood in fish, although it has become clear that some transport mechanisms may be conserved in vertebrate evolution (Boyer *et al.* 1976; Cai *et al.* 2000; Cai *et al.* 2001; Cai *et al.* 2002).

The uptake of bile acids in fish has been shown to involve a Na^+-independent carrier system (Fricker *et al.* 1994; Fricker *et al.* 1987; Rabergh *et al.* 1994). Several studies have identified and functionally characterized the uptake of organic ions by OATPs in skate (*Raja erinacea*) liver (Cai *et al.* 2002; Jacquemin *et al.* 1995; Wang *et al.* 2001). These studies suggested that this primitive Skate OATP is most closely related to human OATP. Sulfated bile alcohols, rather than bile acids, are commonly the major constituent of fish bile (Karlaganis *et al.* 1989; Kihira *et al.* 1984; Tammar 1974). Fricker *et al.* (Fricker *et al.* 1997) demonstrated efficient enterohepatic circulation for a

bile alcohol sulfate in fish liver, indicating specific hepato-biliary transport mechanisms for this class of compounds (Fricker *et al.* 1997). Bile alcohols do not appear to share the transport mechanisms identified for bile acids in fish (Fricker *et al.* 1994).

Transport systems involved in the secretion of bile into canaliculi also appear to resemble mammalian systems. MDR transport proteins have been identified in the livers of several fish species (Albertus and Laine 2001; Cai *et al.* 2003; Doi *et al.* 2001; Sturm *et al.* 2001; Tutundjian *et al.* 2002). Several studies indicate that MRPs are also involved in the secretion of fish bile (Cai *et al.* 2003; Rebbeor *et al.* 2000; Sauerborn *et al.* 2004). Ballatori *et al.* (Ballatori *et al.* 2000) provided functional and structural evidence for a BSEP protein analogue in the canalicular membrane of skate liver that transports both bile salts and bile alcohols into biliary canaliculi (Ballatori *et al.* 2000). Rabergh *et al.* (Rabergh *et al.* 1992) demonstrated that toxicants can disrupt hepatobiliary transport processes in Rainbow Trout.

Cholestasis can result from genetic defects, toxins, disease, inflammation and oxidative stress (Trauner M 2005). Reduced expression and function of bile transport proteins are important factors in the development of cholestasis (Elferink 2003; Trauner *et al.* 1998). Structural or functional damage to the hepatocyte and biliary epithelial cells can also contribute to cholestasis. Toxins can effect the formation and secretion of bile, presumably by affecting transport protein expression and function (Trauner *et al.* 1998). Schmitt *et al.* (2000) demonstrated that oxidative stress can lead to cholestasis by reducing the number of functional transporters in the canalicular membrane. These authors concluded that severe glutathione depletion induces cholestasis with a retrieval of MRP2 from the canalicular membrane. The impact of oxidative stress on hepatic

glutathione are reviewed by Lu *et al.* (Lu 1999). Hepatocytes under oxidative stress have a reduced ability to reduce oxidized glutathione (GSSG) to reduced glutathione (GSH), which is essential for several cellular functions. Cellular GSH may become depleted under high levels of oxidative stress. Inflamation can also result in the inhibition of biliary transport proteins due to proinflammatory cytokines in the liver, which also leads to oxidative stress (Spirli *et al.* 2001; Trauner *et al.* 1999). Bile acids that are accumulated in liver during cholestasis damage mitochondria, generate oxidative stress, and can lead to cell death (Palmeira and Rolo 2004; Rolo *et al.* 2004; Sokol *et al.* 1995).

Cholestatis has been shown to induce several compensatory changes aimed at establishing alternative elimination pathways for biliary constituents (Trauner M 2005). A commonly observed compensatory change is the development of additional hepatic biliary ducts (bile duct hyperplasia), which can be induced by several conditions associated with cholestasis (reviewed by LeSage *et al.* 2001). The expression of biliary transport proteins is regulated by various nuclear receptors, which can also initiate compensatory changes in biliary transport systems (Karpen 2002). Such adaptive changes can provide alternative elimination pathways for bile constituents through increased expression of transport proteins and induction of alternative transport proteins in the liver and kidney. Compensatory changes also include increased expression of phase I and II detoxifying enzymes (Trauner M 2005). The adaptations discussed here may help to limit the build-up of toxic biliary constituents, however recent work suggests that they are not sufficient to prevent liver injury during chronic cholestasis (Trauner M 2005; Wagner *et al.* 2004).

While cholestasis and related disease states, such as primary biliary cirrhosis and primary sclerosing cholangitis, are well recognized in rodents and mammals, hepatobiliary biliary related disease states are only beginning to be understood in fish species (Cai *et al.* 2001; Kirby *et al.* 1995; Lorent *et al.* 2004). The underlying mechanisms of toxicologically-induced cholestasis should be further investigated.

References (Appendix A):

Albertus JA, Laine RO. 2001. Enhanced xenobiotic transporter expression in normal teleost hepatocytes: Response to environmental and chemotherapeutic toxins. Journal of Experimental Biology 204(2):217-227.

Arrese M, Ananthanarayanan M. 2004. The bile salt export pump: molecular properties, function and regulation. Pfluegers Archiv European Journal of Physiology 449(2):123-131.

Arrese M, Trauner M. 2003. Molecular aspects of bile formation and cholestasis. Trends in Molecular Medicine 9(12):558-564.

Ballatori N, Rebbeor JF, Connolly GC, Seward DJ, Lenth BE, Henson JH, Sundaram P, Boyer JL. 2000. Bile salt excretion in skate liver is mediated by a functional analog of Bsep/Spgp, the bile salt export pump. American Journal of Physiology 278(1 part 1):G57-G63.

Boyer JL, Schwarz J, Smith N. 1976. Biliary Secretion in Elasmobranchs Part 2 Hepatic Uptake and Biliary Excretion of Organic Anions. American Journal of Physiology 230(4):974-981.

Cai S-Y, Soroka CJ, Ballatori N, Boyer JL. 2003. Molecular characterization of a multidrug resistance-associated protein, Mrp2, from the little skate. American Journal of Physiology 284(1 Part 2):R125-R130.

Cai S-Y, Wang L, Ballatori N, Boyer JL. 2000. Molecular identification and functional characterization of the bile export pump (Bsep) in a marine vertebrate: Evidence that this liver specific transporter is highly conserved in evolution. Hepatology 32(4 Pt. 2):493A.

Cai S-Y, Wang L, Ballatori N, Boyer JL. 2001. Bile salt export pump is highly conserved during vertebrate evolution and its expression is inhibited by PFIC type II mutations. American Journal of Physiology 281(2 Part 1):G316-G322.

Cai S-Y, Wang W, Soroka CJ, Ballatori N, Boyer JL. 2002. An evolutionarily ancient Oatp: Insights into conserved functional domains of these proteins. American Journal of Physiology 282(4 Part 1):G702-G710.

Doi AM, Holmes E, Kleinow KM. 2001. P-glycoprotein in the catfish intestine: Inducibility by xenobiotics and functional properties. Aquatic Toxicology 55(3-4):157-170.

Elferink RO. 2003. Cholestasis. Gut 52(Supplement 2):ii42-ii48.

Ferenci P, Zollner G, Trauner M. 2002. Hepatic transport systems. Journal of Gastroenterology & Hepatology 17(Supplement):S105-S112.

Fricker G, Dubost V, Finsterwald K, Boyer JL. 1994. Characteristics of bile salt uptake into skate hepatocytes. Biochemical Journal 299(3):665-670.

Fricker G, Hugentobler G, Meier PJ, Kurz G, Boyer JL. 1987. Identification of a Single Sinusoidal Bile Salt Uptake System in Skate Liver. American Journal of Physiology 253(6 PART 1):G816-G822.

Fricker G, Wossner R, Drewe J, Fricker R, Boyer JL. 1997. Enterohepatic circulation of scymnol sulfate in an elasmobranch, the little skate (*Raja erinacea*). American Journal of Physiology 273(5 PART 1):G1023-G1030.

Groothuis GMM, Meijer DKF. 1996. Drug traffic in the hepatobiliary system. Journal of Hepatology 24(SUPPL. 1):3-28.

Hampton JA, Lantz RC, Goldblatt PJ, Lauren DJ, Hinton DE. 1988. Functional Units in Rainbow Trout *Salmo Gairdneri* Richardson Liver II. the Biliary System. Anatomical Record 221(2):619-634.

Hinton DE, Couch JA. 1998. Architectural pattern, tissue and cellular morphology in livers of fishes: Relationship to experimentally-induced neoplastic responses. Braunbeck, T., Hinton, D. E., Streit, B., editors; Fish Ecotoxicology. 1998. 141-164. Vol. 86.: Birkhaeuser Verlag, Basel; Birkhaeuser Boston, Inc., Massachusetts.

Jacquemin E, Hagenbuch B, Wolkoff AW, Meier PJ, Boyer JL. 1995. Expression of sodium-independent organic anion uptake systems of skate liver in *Xenopus laevis* oocytes. American Journal of Physiology 268(1 PART 1):G18-G23.

Kanno N, LeSage G, Glaser S, Alpini G. 2001. Regulation of cholangiocyte bicarbonate secretion. American Journal of Physiology 281(3 Part 1):G612-G625.

Karlaganis G, Bradley SE, Boyer JL, Batta AK, Salen G, Egestad B, Sjovall J. 1989. A Bile Alcohol Sulfate as a Major Component in the Bile of the Small Skate *Raja Erinacea*. Journal of Lipid Research 30(3):317-322.

Karpen S. 2002. Nuclear receptor regulation of hepatic function. J Hepatol. 36:832-850.

Kihira K, Akashi Y, Kuroki S, Yanagisawa J, Nakayama F, Hoshita T. 1984. Bile Salts of the Coelacanth *Latimeria Chalumnae*. Journal of Lipid Research 25(12):1330-1336.

Kirby GM, Stalker MJ, Gordon S, Quinn BA, Van Schooten FJ, Hayes MA. 1995. Influences of chronic cholangiohepatitis and cholestasis on hepatic metabolism of benzo(a)pyrene in white suckers (*Catostomus commersoni*) from industrially polluted areas of Lake Ontario. Carcinogenesis 16(12):2923-2929.

LeSage G, Glaser S, Alpini G. 2001. Regulation of cholangiocyte proliferation. Liver 21(2):73-80.

Lorent K, Yeo S-b, Oda T, Chandrasekharappa S, Chitnis A, Matthews RP, Pack M. 2004. Inhibition of Jagged-mediated Notch signaling disrupts zebrafish biliary development and generates multi-organ defects compatible with an Alagille syndrome phenocopy. Development 131(22):5753-5766.

Lu SC. 1999. Regulation of hepatic glutathione synthesis: Current concepts and controversies. FASEB Journal 13(10):1169-1183.

Nathanson MH, Boyer JL. 1991. Mechanisms and Regulation of Bile Secretion. Hepatology 14(3):551-566.

Palmeira CM, Rolo AP. 2004. Mitochondrially-mediated toxicity of bile acids. Toxicology 203(1-3):1-15.

Rabergh CMI, Isomaa B, Eriksson JE. 1992. The resin acids dehydroabietic acid and isopimaric acid inhibit bile acid uptake and perturb potassium transport in isolated hepatocytes from rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology 23(3-4):169-179.

Rabergh CMI, Ziegler K, Iosmaa B, Lipsky MM, Eriksson JE. 1994. Uptake of taurocholic acid and cholic acid in isolated hepatocytes from rainbow trout. American Journal of Physiology 267(3 PART 1):G380-G386.

Rebbeor JF, Connolly GC, Henson JH, Boyer JL, Ballatori N. 2000. ATP-dependent GSH and glutathione S-conjugate transport in skate liver: Role of an Mrp functional homologue. American Journal of Physiology 279(2 Part 1):G417-G425.

Rolo AP, Palmeira CM, Holy JM, Wallace KB. 2004. Role of mitochondrial dysfunction in combined bile acid-induced cytotoxicity: The switch between apoptosis and necrosis. Toxicological Sciences 79(1):196-204.

Sauerborn R, Polancec DS, Zaja R, Smital T. 2004. Identification of the multidrug resistance-associated protein (mrp) related gene in red mullet (*Mullus barbatus*). Marine Environmental Research 58(2-5):199-204.

Schmitt M, Kubitz R, Wettstein M, vom Dahl S, Haeussinger D. 2000. Retrieval of the Mrp2 gene encoded conjugate export pump from the canalicular membrane contributes to cholestasis induced by tert-butyl hydroperoxide and chloro-dinitrobenzene. Biological Chemistry 381(5-6):487-495.

Sokol RJ, Winklhofer-Roob BM, Devereaux MW, McKim JM, Jr. 1995. Generation of Hydroperoxides in Isolated Rat Hepatocytes and Hepatic Mitochondria Exposed to Hydrophobic Bile Acids. Gastroenterology 109(4):1249-1256.

Spirli C, Nathanson MH, Fiorotto R, Duner E, Denson LA, Sanz JM, Di Virgilio F, Okolicsanyi L, Casagrande F, Strazzabosco M. 2001. Proinflammatory cytokines inhibit secretion in rat bile duct epithelium. Gastroenterology 121(1):156-169.

Sturm A, Ziemann C, Hirsch-Ernst KI, Segner H. 2001. Expression and functional activity of P-glycoprotein in cultured hepatocytes from *Oncorhynchus mykiss*. American Journal of Physiology 281(4 Part 2):R1119-R1126.

Tammar AR. 1974. Bile Salts in Fishes. Florkin, Marcel and Bradley T. Scheer (Ed.). Chemical Zoology, Vol. 8. Deuterostomians, Cyclostomes and Fishes. Xxii+682p. Illus. Academic Press 3, Inc.: New York, N.Y., U.S.a.; London, England. 1974. 595-612.

Trauner M, Boyer JL. 2003. Bile salt transporters: Molecular characterization, function, and regulation. Physiological Reviews 83(2):633-671.

Trauner M, Fickert P, Stauber RE. 1999. Inflammation-induced cholestasis. Journal of Gastroenterology & Hepatology 14(10):946-959.

Trauner M, Meier PJ, Boyer JL. 1998. Molecular pathogenesis of cholestasis. New England Journal of Medicine 339(17):1217-1227.

Trauner M WM, Fickert P, Zollner G. 2005. Molecular regulation of hepatobiliary transport systems: clinical implications for understanding and treating cholestasis. J Clin Gastroenterol. 39(4 Suppl 2):S111-24.

Tutundjian R, Cachot J, Leboulenger F, Minier C. 2002. Genetic and immunological characterisation of a multixenobiotic resistance system in the turbot (*Scophthalmus maximus*). Comparative Biochemistry & Physiology. Part B, Biochemistry & Molecular Biology 132B(2):463-471.

Wagner M, Fickert P, Zollner G, Fuchsbichler A, Gumhold J, Silbert D, Zatloukal K, Guo G, Marschall H, Denk H and others. 2004. Role of adaptive hepatobiliary transporter expression and regulation of bile acid synthetic enzymes in MDR2-/- Mice: A model for chronic cholestatic liver disease due to sclerosing cholangitis Journal of Hepatology 40 (Supplement 1):163

Wang W, Seward DJ, Li L, Boyer JL, Ballatori N. 2001. Expression cloning of two genes that together mediate organic solute and steroid transport in the liver of a marine vertebrate. Proceedings of the National Academy of Sciences of the United States of America 98(16):9431-9436.

Appendix B. Summary data on spawning induction and success in white sturgeon fed either control (*ca.* 1.42 µg/g Se) or treatment (*ca.* 34 µg/g Se) diets for approximately six months.

Table B-1. Detailed spawning data for white sturgeon fed either control (*ca.* 1.42 μg/g Se) or treatment (*ca.* 34 μg/g Se) diets for approximately six months.

Date	Fish #[1]	Pit Tag #	Tank #	Spawn Hold Tank #	Cont / Trt	FL (cm)	BW (kg)[2]	K	Egg Diameter	PI	% GVBD Prog	% GVBD Control	Comments
9/26/2001		140A	203		Trt	137.5	22.5	0.87	2.66				
2/12/2002		140A	203		Trt	140.5	25.2	0.91	3.10	0.13	100	0	
3/13/2002		140A	203		Trt	140.5	25.2	0.91	3.10	0.09	100	0	well polar
3/22/2002	T1			123	ovulated and good fertility				about 50% hatch				
9/26/2001		772A	204		Cont	136	23.65	0.94	2.85				
2/12/2002		772A	204		Cont	138.5	24.4	0.92	3.23	0.11	0	0	no pectorals, gonads a little fatty
3/13/2002		772A	204		Cont	138.5	24.4	0.92	3.21	0.09	100	0	
3/22/2002	C1			125	no ovulation (in hindsight yolk not well polarized)								
9/26/2001		594D	204		Cont	138	24.4	0.93	2.88				
2/12/2002		594D	204		Cont	139.5	24.8	0.91	3.28	0.12	57	0	
3/13/2002		594D	204		Cont	139.5	24.8	0.91	3.21	0.10	100	0	some fat in gonads
3/22/2002	C2			126	no ovulation (in hindsight yolk not well polarized)								
9/26/2001		6F06	203		Trt	135.5	23.9	0.96	2.53				
2/12/2002		6F06	203		Trt	139	24.6	0.92	3.15	0.17	90	0	
3/28/2002		6F06	203		Trt	139	24.6	0.92	3.19	0.08	100	0	well polarized
4/5/2002	T2			126	ovulated and good fertility				about 50% hatch				

Date	Fish #[1]	Pit Tag #	Tank #	Spawn Hold Tank #	Cont / Trt	FL (cm)	BW (kg)[2]	K	Egg Diameter	PI	% GVBD Prog	% GVBD Control	Comments
9/26/2001		4F7F	204		Cont	121	19.55	1.10	2.73				
2/12/2002		4F7F	204		Cont	122.5	20.5	1.12	3.21	0.15	67	0	
3/28/2002		4F7F	204		Cont	122.5	20.5	1.12	3.19	0.08	100	0	well polarized
4/5/2002	C3			125	ovulated and good fertility				very poor hatch (<1%)				
9/26/2001		0005	204		Cont	140	25.15	0.92	2.75				
2/12/2002		0005	204		Cont	142	25.8	0.90	3.20	0.15	100	0	
3/13/2002		0005	204		Cont	142	25.8	0.90	3.10	0.12	100	0	
4/9/2002		0005	204		Cont	142	25.8	0.90	3.19	0.09	100	77	well polarized
4/19/2002	C4			125	ovulated								
9/26/2001		6D5C	203		Trt	136	22.9	0.91	2.92				
2/12/2002		6D5C	203		Trt	138	24.1	0.92	3.14	0.13	100	0	no left pectoral
3/13/2002		6D5C	203		Trt	138	24.1	0.92	3.11	0.12	100	0	no left pectoral
4/9/2002		6D5C	203		Trt	138	24.1	0.92	3.21	0.09	100	0	well polarized
4/19/2002	T3			126	partial ovulation								
9/26/2001		4304	203		Trt	133	24.05	1.02	2.71				
2/12/2002		4304	203		Trt	136.5	25.9	1.02	3.09	0.15	0	0	
3/13/2002		4304	203		Trt	136.5	25.9	1.02	3.08	0.09	0	0	all prog at PI = 0.0
3/28/2002		4304	203		Trt	136.5	25.9	1.02	3.14	0.10	0	0	all prog at PI = 0.0, med polar.
4/25/2002		4304	203		Trt	136.5	25.9	1.02	3.24	0.07	100	0	well polarized
5/3/2002	T4			125	ovulated, hatch almost nothing								

Date	Fish #[1]	Pit Tag #	Tank #	Spawn Hold Tank #	Cont / Trt	FL (cm)	BW (kg)[2]	K	Egg Diameter	PI	% GVBD Prog	% GVBD Control	Comments
9/26/2001		6F36	204		Cont	127.5	19.6	0.95	2.74				
2/12/2002		6F36	204		Cont	130	20.8	0.95	3.12	0.16	0	0	
3/28/2002		6F36	204		Cont	130	20.8	0.95	3.12	0.13	77	0	med polarization
4/25/2002		6F36	204		Cont	130	20.8	0.95	3.14	0.08	100	0	well polarized
5/3/2002	C5			126	ovulated, hatch about 40%								
9/26/2001		3A67	204		Cont	129	17.85	0.83	2.90				
2/12/2002		3A67	204		Cont	131	21.2	0.94	3.14	0.16	0	0	
3/28/2002		3A67	204		Cont	131	21.2	0.94	3.10	0.16	0	0	all prog at PI = 0.0
4/25/2002		3A67	204		Cont	131	21.2	0.94	atretic				
6/4/2002	C6						necropsied						
9/26/2001		6D74	204		Cont	140.5	24.05	0.87	2.86				
2/12/2002		6D74	204		Cont	142	25.8	0.90	3.17	0.13	0	0	
3/13/2002		6D74	204		Cont	142	25.8	0.90	3.12	0.11	100	0	
4/9/2002		6D74	204		Cont	142	25.8	0.90	3.10	0.12	100	0	low-med polarization
5/8/2002									atretic				
6/4/2002	C7						necropsied						
9/26/2001		1D75	203		Trt	147	27.4	0.86	2.75				
2/12/2002		1D75	203		Trt	149	28.8	0.87	3.09	0.13	4	0	left pectoral curled
3/13/2002		1D75	203		Trt	149	28.8	0.87	3.09	0.12	0	0	very fatty gonad
4/9/2002		1D75	203		Trt	149	28.8	0.87	3.10	0.11	77	0	low-med polarization
5/8/2002		1D75	203		Trt	149	28.8	0.87	3.10	0.15	77	0	low-med polarization
5/17/2002	T6						necropsied						

Date	Fish #[1]	Pit Tag #	Tank #	Spawn Hold Tank #	Cont / Trt	FL (cm)	BW (kg)[2]	K	Egg Diameter	PI	% GVBD Prog	% GVBD Control	Comments
9/26/2001		192E	203		Trt	129	21	0.98	2.53				
2/12/2002		192E	203		Trt	134.5	22.7	0.93	3.17	0.18	18	0	
4/9/2002		192E	203		Trt	134.5	22.7	0.93	3.23	0.15	100	0	
5/8/2002		192E	203		Trt	134.5	22.7	0.93	3.22	0.15	100	0	
5/17/2002	T5			125		injected, no ovulation, necropsied							
9/26/2001		6D0E	203		Trt	148.5	30.4	0.93	2.88				
2/12/2002		6D0E	203		Trt	148.5	30.4	0.93	3.25	0.19	0	0	
4/9/2002		6D0E	203		Trt	148.5	30.4	0.93	3.20	0.17	0	0	
5/8/2002		6D0E	203		Trt	148.5	30.4	0.93	3.26	0.17	0	0	
5/29/2002		6D0E	203		Trt	148.5	30.4	0.93	3.23	0.17	0	0	
6/4/2002	T8						necropsied						
9/26/2001		293B	203		Trt	128	18.9	0.90	2.35				
2/12/2002		293B	203		Trt	128	18.9	0.90	3.20	0.22	0	0	
4/9/2002		293B	203		Trt	128	18.9	0.90	3.25	0.18	0	0	
5/8/2002		293B	203		Trt	128	18.9	0.90	3.27	0.17	0	0	
5/29/2002		293B	203		Trt	128	18.9	0.90	3.25	0.17	0	0	
6/4/2002	T7						necropsied						

[1]Control female number C8 (PIT Tag 4519) was atretic on 2/12/02 and later died due to technical error (escaped tank through a rip in the tank covering).

[2]Body weight for 2/12/02 estimated from fork length.

Table B-2. Summary of spawning induction and success in white sturgeon fed either control (*ca.* 1.42 μg/g Se) or treatment (*ca.* 34 μg/g Se) diets for approximately six months.

	T1	C1	C2	T2	C3	C4	T3	T4	C5	T5
PIT Tag#	140A	772A	594D	6F06	4F7F	0005	6D5C	4304	6F36	192E
1st Inj Date	3/20/2002	3/20/2002	3/20/2002	4/3/2002	4/3/2002	4/17/2002	4/17/2002	5/1/2002	5/1/2002	5/15/2002
1st Inj Time	10:15pm	10:20pm	10:25pm	9:50pm	9:50pm	10pm	10pm	10pm	10pm	9pm
2nd Inj Date	3/21/2002	3/21/2002	3/21/2002	4/4/2002	4/4/2002	4/18/2002	4/18/2002	5/2/2002	5/2/2002	5/16/2002
2nd Inj Time	9:57am	10:00am	10:05am	10am	10am	10am	10am	10am	10am	9am
1st Inj Temp (°C)	14.8	15.0	13.0	14.6	14.6	14.5	14.8	14.7	14.6	15.1
2nd Inj Temp (°C)	15.3	15.2	14.0	14.7	14.7	15.1	15.3	15.3	15.3	15.6
Ovul Temp (°C)	15.0			15.4	15.3	15.3	15.4	15.5	15.5	
Latency (h)	19.0			21.0	21.0	23.0	25.5	24.5	24.0	
Ovulation %	100	no ovul	no ovul	100	100	100	partial (40-50%)	100	100	no ovul
Volume Eggs (mls)	3250	(sampled follicles)	(sampled follicles)	3400	2550	4350	950	2200	2400	(sampled follicles)
# eggs/ml	31.15	(100% gvbd)	(20% gvbd)	24.95	28.8	31.55	33.65	30.3	30.45	(no gvbd)
# Eggs Collected (est fecundity)	101238			84830	73440	137243	31968	66660	73080	
Used pooled milt for fertilization from # of males	2			3	3	4	4	4	4	